Pharmacological and nutritional targeting of voltage-gated sodium channels in the treatment of cancers

Osbaldo LOPEZ-CHARCAS[1], Piyasuda PUKKANASUT[2], Sadanandan E. VELU[2], William J. BRACKENBURY[3], Tim G. HALES[4], Pierre BESSON[1], Juan Carlos GOMORA[5], Sébastien ROGER[1,6#]

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[1], Université de Tours, EA4245 Transplantation, Immunologie, Inflammation, Faculté de Médecine de Tours, 10 Boulevard Tonnellé, 37032 Tours, France ; sebastien.roger@univ-tours.fr; pierre.besson@univ-tours.fr; osbaldo.lopez@univ-tours.fr

[2], Department of Chemistry, The University of Alabama at Birmingham, CHEM 280. 901, 14th Street S, Birmingham, AL 35294, USA, svelu@uab.edu; (205) 975-2478.

[3], Department of Biology, York Biomedical Research Institute, University of York, Heslington, York, YO10 5DD, United Kingdom. william.brackenbury@york.ac.uk +44 (0) 1904 328 284

[4], Institute of Academic Anaesthesia, Division of Systems Medicine, School of Medicine, the University of Dundee, DD1 9SY, Dundee, United Kingdom, t.g.hales@dundee.ac.uk; +44 (0)1382 383443

[5], Instituto de Fisiología Celular, Circuito Exterior s/n Ciudad Universitaria, Universidad Nacional Autónoma de México, Mexico City, 04510 México; jgomora@ifc.unam.mx; + (5255) 5622-5752

[6], Institut Universitaire de France, 75005 Paris, France

#, Correspondence to (lead contact):

Dr Sébastien Roger,

Université de Tours, EA4245 Transplantation, Immunologie, Inflammation, Faculté de Médecine de Tours, 10 Boulevard Tonnellé, 37032 Tours, France.

E-mail : sebastien.roger@univ-tours.fr ; Phone: +33 (0)2 47 36 61 30

Summary

Voltage-gated sodium (Na_V) channels, initially characterized in excitable cells, have been shown to be aberrantly expressed in non-excitable cancer tissues and cells from epithelial origins such as in breast, lung, prostate, colon, cervix, while they are not expressed in cognate non-cancer tissues. Their activity was demonstrated to promote aggressive and invasive potencies of cancer cells, both *in vitro* and *in vivo*, while their deregulated expression in cancer tissues has been associated with metastatic progression and cancer-related death. This review proposes Na_V channels as pharmacological targets for anticancer treatments providing opportunities for repurposing existing Na_V-inhibitors or developing new pharmacological and nutritional interventions.

Introduction

Voltage-gated sodium (Na_V) channels, composed of pore-forming $Na_V\alpha$ and auxiliary $Na_V\beta$ subunits, were initially characterized in excitable cells in which they are responsible for the triggering and the propagation of action potentials. Their physiological activity, through a transient depolarizing inward sodium current in cell types such as cardiomyocytes, skeletal muscle cells or neurons, is well characterized as being responsible for the initiation of excitation-contraction, excitation-secretion or excitation-expression couplings. As such, these ion channels are critical in numerous physiological functions and mutations in their encoding genes, as well as dysregulation of their activity, may lead to serious pathologies called "sodium channelopathies". Na_V channels are targets for multiple inhibitory molecules that are FDA-approved and clinically used in the treatment of pathologies such as cardiac angina or arrhythmias, epilepsies, chronic pain, or in anesthesiology.

While the activity of Na_V channels has been characterized about 70 years ago, recent data obtained in the past five years have shed light on the protein structure, arrangement and functioning at the molecular level. Indeed, the activity of Na_V channels, *i.e*. sodium currents (I_{Na}), was first recorded by Hodgkin and Huxley in 1952 from the squid giant axon, using the voltage-clamp technique. These pioneering experiments led to the ionic theory of membrane excitability (Hodgkin and Huxley, 1952). However, at that time the structure of Na_V channels was not known and the first evidence of molecular properties came to light at the beginning of the 1980's with the identification of the channel proteins using radio-labeled-neurotoxins highly selective for Na_V channels in combination with protein solubilization and purification methods (Agnew et al., 1980, Beneski and Catterall, 1980). Further structural insights into Na_V channels were obtained by cloning and screening of cDNA libraries leading to the discovery of the amino acid sequence for these proteins and allowing for modelling of secondary structures based on aliphatic profiles (Noda et al., 1986, Noda et al., 1984). These seminal studies allowed development of a model in which the pore-forming Na_V principal subunit in eukaryotes, later called $Na_V\alpha$-subunit, is composed of a single polypeptide chain of approximately 260 kDa containing four repeated homologous domains (I-IV) of 6 transmembrane segments (S1-S6). This protein was identified to interact with one or two single-span transmembrane auxiliary subunits, called $Na_V\beta$-subunits (30-40 kDa), initially characterized as bringing regulatory functions (Isom et al., 1994, Isom et al., 1992) to the macromolecular complex of eukaryotic Na_V channels (Brackenbury and Isom, 2011, Catterall, 2000).

Interestingly, understanding of protein organization and function at the molecular level substantially progressed very recently with the use of X-ray protein crystallography and cryo-electron microscopy,

first studying tetrameric prokaryotic channels sharing approximately 25-30% sequence identity with human channels. Thus information regarding the voltage-dependent gating, ion selectivity, drug binding, open, closed and inactivated states were acquired from the *Arcobacter butzleri* (NavAb) channel (Payandeh et al., 2012, Payandeh et al., 2011) (Lenaeus et al., 2017). The high-resolution crystal structure of the complete voltage-gated sodium channel (Na_VMs) from *Magnetococcus marinus* was obtained in the activated open state, associated with electrophysiological recordings (Sula et al., 2017). Recently, structural data obtained for Na_VAb provided a complete gating mechanism for voltage sensor function, pore opening, and the activation-gate (Wisedchaisri et al., 2019). The first near-atomic resolution structure of a monomeric eukaryotic channel came from the recent study of the Na_VPaS from the American cockroach, while structures of human voltage-gated sodium channel $Na_V1.2$, $Na_V1.4$ and $Na_V1.7$, in complex with β-subunits were recently published (Pan et al., 2018, Pan et al., 2019, Shen et al., 2019). These structural data provide important insight into mechanisms underlying Na_V channelopathies and for drug discovery. Briefly, each domain presents two functional modules: S1-S4 segments comprise the voltage-sensor module (VSM) while sections (S5 - P loop - S6) constitute the pore-forming module (PM). The positively charged arginine and lysine residues, positioned at every third residue within each S4 segment in the voltage-sensor module, sense changes in the transmembrane potential and transform this electrical stimulus into a fast conformational change of the pore-forming module, allowing the opening of the conductive pore, permitting Na^+ influx. One to two milliseconds after pore opening, another fast voltage-dependent mechanism happens in the Na_V channel protein, occluding Na^+ influx, a process known as fast inactivation.

Another recent study has challenged the initial paradigms by which functional channels contain a monomeric $Na_V\alpha$ subunit. Indeed, $Na_V\alpha$-subunits appear to assemble as dimers and this physical interaction permits a coupled gating mechanism (Clatot et al., 2017). In humans, there are nine different genes encoding for $Na_V\alpha$-subunits, four of them clustered on chromosome 2: *SCN1A* ($Na_V1.1$), *SCN2A* ($Na_V1.2$), *SCN3A* ($Na_V1.3$) and *SCN9A* ($Na_V1.7$); three others located on chromosome 3: *SCN5A* ($Na_V1.5$), *SCN10A* ($Na_V1.8$) and *SCN11A* ($Na_V1.9$); and two more located on chromosome 12 and 17: *SCN8A* ($Na_V1.6$) and *SCN4A* ($Na_V1.4$), respectively (Goldin, 2002). The amino acid sequence homology among Na_V channels subtypes is higher than 70% in the transmembrane and extracellular motifs so that there are no distinct subfamilies, nonetheless, some of the isoforms are more closely related to each other, sharing chromosomal localization and sensitivity to tetrodotoxin (TTX), which has been explained by the early genomic duplication during the evolution of Na_V channel genes. To date, four genes have been identified for $Na_V\beta$-subunits, one on chromosome 19: *SCN1B* (encoding for the two splicing variants, transmembrane $Na_V\beta1$ and soluble $Na_V\beta1B$) and the other three on chromosome 11:

SCN2B ($Na_V\beta2$), *SCN3B* ($Na_V\beta3$) and *SCN4B* ($Na_V\beta4$). The structure of $Na_V\beta$-subunit is comprised of a N-terminal extracellular immunoglobulin-like domain, followed by an extracellular juxtamembrane region, a single transmembrane segment and a 34-44 amino acid-length intracellular domain; except for the splice variant $Na_V\beta1B$, which is expressed as a soluble macromolecule (Brackenbury and Isom, 2011). Furthermore, it is worth noting that $Na_V\beta$-subunits not only influence $Na_V\alpha$-subunit trafficking and biophysical modulation but they have also been experimentally shown to act as cell adhesion molecules (CAMs), participating in both homophilic and heterophilic interactions, with contactin, N-cadherin, NrCAM, several types of neurofascin and tenascin being the main binding proteins (Isom, 2002, Bouza and Isom, 2018).

$Na_V\alpha$ and $Na_V\beta$ subunits are differentially and developmentally expressed in several tissues and cell types (Black and Waxman, 2013, Roger et al., 2015). Initial studies identified Na_V to be distributed in excitable tissues such as in mammalian central and peripheral nervous systems as well as in skeletal and cardiac muscle. The central nervous system channels mainly comprise the $Na_V1.1$, $Na_V1.2$, $Na_V1.3$ and $Na_V1.6$ isoforms; while the peripheral nervous system channels include $Na_V1.7$, $Na_V1.8$ and $Na_V1.9$. $Na_V1.4$ and $Na_V1.5$ were characterized as being the main skeletal and heart muscle isoforms, respectively (Goldin, 2001), while they also have been identified to be expressed the brain and in the dorsal root ganglion (Wang et al., 2008, Wang et al., 2009, Wang et al., 2018a, Wang et al., 2018b, Bergareche et al., 2015). Therefore, the expression of Na_V has long been considered as the hallmark of excitable cells. Again, this paradigm has recently changed with the identification of expression (mRNA and protein) and sometimes activity at the plasma membrane (transient sodium currents) in non-excitable tissues and cells such as in chondrocytes, endothelial cells, microglia, astrocytes, fibroblasts, keratinocytes, islet β-cells, red blood cells, T-lymphocytes, dendritic cells and macrophages, among others, in which the biological role and subcellular distribution of Na_Vs is still elusive (Black and Waxman, 2013, Roger et al., 2015) .

Recently, it has emerged that $Na_V\alpha$ channels, as well as auxiliary $Na_V\beta$ subunits, are aberrantly expressed in non-excitable cancer tissues and cells from different epithelial origins such as breast, lung, prostate, colon, cervix, while they are not expressed in cognate non-cancer tissues. Their expression in carcinoma cells has been associated with cancer progression, suggesting that they could serve as cancer markers and prognostic factors. The expression and activity of Na_V channels was shown to promote pro-cancerous properties and, importantly, to contribute to disease progression, in both *in vitro* and *in vivo* models. Recent studies shed light on the signaling pathways that are under the control of these channel proteins, coupling membrane activity to cellular properties. Furthermore, the inhibition of Na_V channel activity, using small synthetic compounds and FDA-approved drugs as well as

with natural dietary compounds potentially opens up new therapeutic strategies. In this review, we summarize current knowledge on the expression of Na_V channels in cancers, highlight the signaling pathways involved, and discuss pharmacological and nutritional strategies that represent opportunities for novel anticancer treatments.

I- The aberrant expression of $Na_V\alpha$ and $Na_V\beta$ subunits in cancers

The expression of voltage-gated sodium channel subunits, both $Na_V\alpha$ and $Na_V\beta$, has been reported to be altered in several types of cancer. The channel subunits have been detected by molecular biology and biochemical techniques, and in multiple cancer types Na_V activity at the plasma membrane, *i.e*. I_{Na}, could be recorded. Most subunits have been shown to be up-regulated in cancers, while some of them appear to be downregulated. This aberrant expression has been correlated with oncogenic properties in both *in vitro* and *in vivo* models of cancer, mostly in solid tumours including carcinomas (Figure 1).

a- $Na_V\alpha$ and $Na_V\beta$ subunits in Prostate cancer

While early works assessing ion channel activity identified sodium currents in small-cell lung cancer cells (Pancrazio et al., 1989), the first report of the direct contribution of Na_V channels in cancer properties came from work performed in prostate cancer (PCa) cells by Prof. M. Djamgoz' group almost 25 years ago. This first study was performed in two rat prostatic tumour cell lines in which they found a differential expression of voltage-activated Na^+ currents: highly metastatic Mat Ly-Lu cells expressed Na^+ currents, whereas weakly metastatic AT-2 cell, did not express this type of current. For the first time, the functional relevance of the Na^+ current was demonstrated: blocking I_{Na} with nanomolar concentrations of TTX reduced by ~30% the invasiveness of the highly metastatic Mat-Ly-Lu cells (Grimes et al., 1995). Further evidence showed that the $Na_V1.7$ pore-forming subunit, encoded by the *SCN9A* gene, was overexpressed in highly metastatic human and rat prostate cell lines in comparison with weakly metastatic cell lines (Diss et al., 2001). Later, this observation was confirmed *in vivo* by showing that $Na_V1.7$ was overexpressed approximately 20 times in PCa biopsies versus non-cancer samples (Diss et al., 2005). Finally, in a rat model, inoculation with highly metastatic Mat-Ly-Lu cells promotes prostate cancer metastasis *in vivo*, and blockade of Na_V in primary tumours with TTX (Yildirim et al., 2012) or ranolazine (Bugan et al., 2019) reduced lung metastases by 40% and 63%, respectively. However, most of these studies were performed in cell lines and there are no systematic studies that

show a positive correlation between mRNA/protein of $Na_V1.7$ upregulation and human PCa patient samples.

Concerning $Na_V\beta$ subunits, it was shown that $Na_V\beta1$ mRNA was the most highly expressed in strongly invasive compared to weakly invasive PCa cell lines (Diss et al., 2008). However, there was no significant difference in the expression of $Na_V\beta1$, or any other $Na_V\beta$ subunits, between cancer and non-cancer human prostate specimens (Diss et al., 2008). $Na_V\beta2$ has been proposed to participate in prostate cancer biology. Indeed, the first experimental observation showed that the overexpression of $Na_V\beta2$, fused to GFP, was properly localized at the plasma membrane of weakly metastatic LNCaP cells inducing morphological changes consistent with a bipolar and elongated migratory phenotype (Jansson et al., 2012). These changes were accompanied by an increase in cell migration and Matrigel invasion *in vitro*, although there was no significant change in cell proliferation. However, the *in vivo* properties of LNCaP cells overexpressing the $Na_V\beta2$ subunit were quite unexpected as subcutaneous tumour volume was drastically reduced compared to the control group (Jansson et al., 2012). Such contrasting effects could imply different mechanisms involved in tumour formation, growth and metastatic behaviour. A novel *ex vivo* organotypic spinal cord co-culture with LNCaP cells revealed that overexpression of $Na_V\beta2$ enhanced the association of PCa cells with nerve axons (Jansson et al., 2014). Furthermore, overexpression of $Na_V\beta2$ enhanced PCa cell migration, invasion, and growth, in the presence of the neuronal CAM laminin, suggesting that the $Na_V\beta2$ subunit may mediate metastatic behaviour through association with neural substrates (Jansson et al., 2014).

b- $Na_V\alpha$ and $Na_V\beta$ subunits in breast and colorectal cancer

Breast cancer (BCa) is the most lethal female cancer worldwide (Bray et al., 2018) and colorectal cancer (CRCa) is the third most commonly diagnosed cancer (Arnold et al., 2017). The incidence of these cancers is gradually increasing, thus representing a serious global health problem. The main cause of patient mortality from these two types of cancers, as for the majority of carcinomas, is the development of metastases in distant organs, following the dissemination of cancer cells from the primary tumour (Figure 1).

Multiple studies have investigated the expression of Na_V channels and their contribution to tumour progression and metastasis in BCa and CRCa. Knowledge about signalling pathways and cellular mechanisms induced by Na_V channels have been mostly acquired from these cancers. In both cases, the major isoform identified was $Na_V1.5$, encoded by the *SCN5A* gene. In BCa samples, $Na_V1.5$ is

overexpressed as compared to normal tissues (Fraser et al., 2005). A high expression was correlated with cancer recurrence, metastasis development and reduced patient survival (Yang et al., 2012). Most of the mechanistic studies in BCa have been performed in human cancer cell lines like MDAMB-231 (highly metastatic) and compared with weakly metastatic cell lines like MCF-7. It has been shown initially shown that MDA-MB-231 express a TTX-resistant Na^+ current, lacking in MCF-7 cells (Roger et al., 2003), which is encoded by a neonatal splice variant of the *SCN5A* gene (Fraser et al., 2005). This neonatal variant is due to a switch from adult exon 6B to foetal exon 6A, which are mutually exclusive and encode for a part of the voltage sensor, segments 3 and 4 located in the domain I of the channel. Therefore, these two variants, called $hNa_V1.5$ and $hNa_V1.5e$ for the adult and the neonatal channels respectively, show different electrophysiological properties in terms of voltage-sensitivity and current kinetics (Murphy et al., 2012, Onkal et al., 2008). These changes result in a greater Na^+ influx for neonatal $hNa_V1.5e$ (Onkal et al., 2008). In the heart, splicing of *SCN5A* is developmentally regulated, such that the neonatal exon 6A is rapidly replaced by the "adult" exon 6B after birth (Murphy et al., 2012) and molecular determinants explaining the abnormal expression of hNaV1.5e in cancer cells have not been identified so far. Nevertheless, the inhibition of channel activity, by either pharmacological (TTX, ranolazine and phenytoin) or molecular (siRNA and inhibitory antibody) approaches, have shown its contribution to migration and invasion of BCa cell lines (Roger et al., 2003, Fraser et al., 2005, Brackenbury et al., 2007, Driffort et al., 2014, Yang et al., 2012). There has also been some significant progress into uncovering the mechanisms underlying the promotion of invasiveness behaviour by $Na_V1.5$. Experimental evidence suggested that $Na_V1.5$ induces pro-migratory and pro-invasive properties through a persistent activity at the membrane potential called "window current", and a correlated depolarisation of the membrane voltage of breast cancer cells. Particularly, $Na_V1.5$ activity induced the allosteric modulation of the Na^+-H^+ exchanger type 1 (NHE-1), resulting in an increased activity leading to the acidification of the extracellular space, thus favouring the pH-dependent activity of proteolytic cysteine cathepsins (Gillet et al., 2009, Brisson et al., 2011). In addition, $Na_V1.5$ expression and activity were demonstrated to increase Src kinase activity, which promotes the acquisition of an invasive morphology (invadopodia) in MDA-MB-231 cells. Taken together, these observations indicate that $Na_V1.5$ promotes invadopodia activity of breast cancer cells and the invasion of the surrounding ECM (Brisson et al., 2013) (Figure 1). Recently, $Na_V1.5$ was identified as importantly promoting the epithelial-to-mesenchymal transition (EMT) and cancer cell invasiveness through the regulation of the Salt-inducible kinase 1 (SIK1) (Gradek et al., 2019). Furthermore, $Na_V1.5$ activates the small GTPase Rac1 by sustaining a plasma membrane depolarization, which as a regulator of activation, induces cytoskeletal reorganization and cellular migration (Yang et al., 2020). In addition, *in vivo* experiments have shown that $Na_V1.5$ activity promotes

metastasis in immunodeficient mice (Driffort et al., 2014, Nelson et al., 2015a, Nelson et al., 2015b). $Na_V1.5$ activity also increases MMP9 expression and reduces apoptosis in primary tumours *in vivo* (Nelson et al., 2015b).

The *SCN5A* gene and its protein product the $Na_V1.5$ channel have also been shown to be overexpressed in colorectal cancer biopsies, as compared to non-cancer samples (House et al., 2010). $Na_V1.5$ was found to be expressed at the plasma membrane of tumour cells, and its activity (I_{Na}) was recorded several carcinoma cell lines (mainly SW-480, SW-620 and HT-29) (House et al., 2010). In colon cancer cells, $Na_V1.5$ activity promotes cancer cell invasion *in vitro*, in both 2- and 3-dimension models and regulates a network of invasion-promoting genes via modulation of the PKA/ERK/cJUN/ELK-1/ETS-1 transcriptional pathway (House et al., 2010, House et al., 2015, Poisson et al., 2020) (Figure 2). It was shown that, similar to BCa, the neonatal exon 6A splice variant of the $Na_V1.5$ isoform has a predominant contribution to the invasiveness of CRCa cell lines (Guzel et al., 2019), even though both adult $hNa_V1.5$ and neonatal $hNa_V1.5e$ splice variants could be detected (Baptista-Hon et al., 2014).

A study reported the downregulation of the *SCN9A* gene, encoding for $Na_V1.7$, in CRCa (Pan et al., 2017). In this study, authors analysed genes differentially expressed in CRCa utilizing three Gene Expression Omnibus (GEO) data sets. By screening 46 biomarkers associated with cancer proliferation, drug-resistance and metastasis, *i.e.*, genes closely associated to patient overall survival, they proposed a risk score with high prognostic value based on the expression of five genes: *MET* (MET proto-oncogene and receptor tyrosine kinase), *CPM* (carboxypeptidase M), *SHMT2* (serine hydroxymethyltransferase 2), *GUCA2B* (guanylate cyclase activator 2B), and *SCN9A*. *MET*, *SHMT2* were up-regulated while *CPM*, *GUCA2B* and *SCN9A* were down-regulated. Interestingly, this observation was confirmed in the human protein atlas immunohistochemistry database (www.proteinatlas.org), as the staining for $Na_V1.7$ was lower in some CRCa sample tissues (Pan et al., 2017). There are also reports indicating the downregulation of $Na_V1.6$, encoded by the *SCN8A* gene, in CRCa (Igci et al., 2015). Tumour samples from CRCa patients exhibited reduced expression of $Na_V1.6$ compared with paired tumour-surrounding normal tissues. *SCN8A* mRNA levels, analysed by real-time qPCR, were significantly lower in tumour tissues and in patients aged below 45 years. Results also reveal a relationship between *SCN8A* expression, gender, grade of CRCa, tumour location and histopathological classification (Igci et al., 2015). On the contrary, $Na_V1.6$ protein was highly expressed in metastatic lymph nodes from CRCa patients (Lin et al., 2019). While the reduced expression of *SCN8A*, encoding for $Na_V1.6$, and *SCN9A*, encoding for $Na_V1.7$ might harbour predictive values in CRCa, we are still missing clear information to assert whether they have a role, either causative or consecutive, in the carcinogenesis or whether their expression dysregulation is only correlative to cancer transformation

or progression. Indeed, the functional activity of $Na_V1.6$ and/or $Na_V1.7$ at the plasma membrane of colorectal non-cancer or cancer cells has not been demonstrated so far. Furthermore, it cannot be excluded that these channels might be expressed in intracellular compartments, in which they might play diverse functions. Eventually, it is not clear at the moment whether these changes in expression levels concern epithelial cells, or non-epithelial cells in the colorectal tract, such as immune cells which are key protagonists in colorectal carcinogenesis. As such, the participation of *SCN8A* and *SCN9A* in CRCa biology will require further studies.

The expression of $Na_V\beta$ subunits has been studied in BCa and in CRCa. Some $Na_V\beta$ have been shown to be up-regulated while others are down-regulated in cancer tissues, and mostly these changes appear to correlate with the metastatic behaviour of cancer cells, in particular with cell migration and invasion. Most research performed so far studying the role of $Na_V\beta$ subunits in metastatic behaviour has been undertaken in BCa. Originally, it was shown that $Na_V\beta1$ was more abundantly expressed in the weakly metastatic MCF-7 than in the highly metastatic MDA-MB-231 cell line. Interestingly, when MCF-7 cells were transfected with specific siRNA directed against $Na_V\beta1$, cell adhesion was reduced by 35%, while migration was increased by 121%. In contrast, stable expression of $Na_V\beta1$ in MDA-MB-231 cells increased process length and adhesion while reducing lateral motility and proliferation. Thus, $Na_V\beta1$ was proposed to act as a cell adhesion molecule in BCa cells, negatively controlling cellular migration (Chioni et al., 2009). Later, it was found that $Na_V\beta1$ was the $Na_V\beta$ subunit most expressed in BCa and was up-regulated (both mRNA and protein) in BCa biopsies, compared with normal breast tissue (Nelson et al., 2014, Bon et al., 2016). More importantly, by using a xenograft model of BCa, it was shown that $Na_V\beta1$ overexpression increased tumour growth, metastasis, and vascularization, while decreasing apoptosis in the primary tumours. Therefore, this study was the first showing the functional role for $Na_V\beta1$ in tumour growth and metastasis *in vivo* (Nelson et al., 2014). Consistent with these results, the use of siRNA to specifically target $Na_V\beta1$ expression in MDA-MB-231 cells inhibited cancer cell invasion (Bon et al., 2016).

The participation of $Na_V\beta3$ in tumorigenesis process is poorly understood. Two missense mutations have been identified in the *SCN3B* gene in high grade metastatic colorectal cancer biopsies (Sjoblom et al., 2006). The first report suggested that non-mutated $Na_V\beta3$ mediates a p53-dependent apoptotic pathway in Saos-2, a bone osteosarcoma cell line, after DNA damage (Adachi et al., 2004). In agreement with this, the *SCN3B* gene is not expressed in highly invasive MDA-MB-231 breast cancer cells (Gillet et al., 2009) or weakly invasive MCF-7 cells (Chioni et al., 2009). In non-tumour breast samples, *SCN3B*

expression was the lowest among all $Na_V\beta$ encoding genes, and was still significantly reduced in cancer samples (Bon et al., 2016).

The *SCN4B* gene was shown to play a critical role as a metastasis-suppressor gene in BCa (Bon et al., 2016). In this study *SCN4B* mRNA appeared to be significantly expressed in normal breast, colon, rectum, lung and prostate but consistently downregulated in cancer samples. Furthermore, $Na_V\beta4$ protein was expressed in normal epithelial cells but significantly reduced in BCa biopsies, especially in high-grade primary and metastatic tumours. *In vitro* experiments showed that reducing $Na_V\beta4$ expression potentiates cell migration and invasiveness though an increase in RhoA activity and the acquisition of a hybrid mesenchymal-amoeboid aggressive phenotype. This effect was independent of $Na_V\alpha$ channel activity and was prevented by overexpression of the intracellular C-terminus of $Na_V\beta4$. On the contrary, *SCN4B* overexpression reduced cancer cell invasiveness and tumour progression. The findings are in line with previous observations showing decreased levels of *SCN4B* in invasive versus non-invasive PCa cells (Diss et al., 2008). Interestingly, a recent study identified dysregulated miRNA in CRCa and reported an increased miR-424-5p expression in tumour samples that was associated with poor prognosis (Dai et al., 2020). miR-424-5p was found to be elevated in the peripheral blood of CRCa patients, most probably secreted in tumour exosomes. In this study, it was demonstrated that overexpression of *SCN4B* inhibited HT-29 CRCa cell proliferation, migration and invasion and expression of *SCN4B* was directly inhibited by miR-424-5p (Dai et al., 2020). These results support the tumour-suppressor role of *SCN4B* in CRCa and identified miR-424-5p as a regulator of its expression in tumours.

c- $Na_V\alpha$ and $Na_V\beta$ subunits in Lung cancer

There are two subtypes of lung cancer, small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Early works assessing ion channel activity have been undertaken in small-cell lung cancer cells. In these pioneering works, I_{Na} was initially recorded in human H146, H69 and H128 small-cell lung cancer cell lines (Pancrazio et al., 1989), although this study did not relate the presence of Na_V currents to lung tumorigenesis process itself. These currents were most probably due to TTX-sensitive channels since they were fully inhibited by 5 µM TTX. While neither the molecular characterization of the channels nor their biological role were described, they were proposed to participate in a "neuroendocrine-like" tumour cell phenotype (Pancrazio et al., 1989). Later, it was shown that these sodium currents were actually able to participate in the generation of action potentials in H146 SCLC cells, thus supporting this idea. Interestingly, 5 µM TTX abolished these action potentials, which implies

the contribution of a TTX-resistant current (Blandino et al., 1995). In fact, H146 cells, sodium currents demonstrated an IC_{50} to TTX of 215 nM, leading the authors to indicated that Na_V channels were weakly TTX-sensitive. However, this concentration is too high to consider the channels to belong to the TTX-S category but too low to belong to TTX-R isoforms. The most likely explanation for this would be the expression of a population of different isoforms of Na_V at the plasma membrane of cells, thus leading to an apparent IC_{50} that would be intermediate between TTX-S and TTX-R. Nevertheless, it cannot be excluded the possibility that Na_V channels expressed in these cells are variants (splice-variants or polymorphism variants) showing specific pharmacological properties.

The hypothesis of the role of $Na_V\alpha$ in the acquisition of a neuroendocrine phenotype was also proposed by M. Djamgoz's team (Onganer et al., 2005). Unexpectedly, in this later study, the endocytic activity of SCLC cells was inhibited by using lower nanomolar concentrations of TTX, suggesting the participation of TTX-sensitive sodium channels in these SCLC cells. In addition, they found mRNA encoding for $Na_v1.3$, $Na_v1.5$ and $Na_v1.6$ in H69, H209 and H510 cell lines. The latter also showed the additional presence of $Na_v1.9$ mRNA. Thus, it remains to be elucidated which Na_v subunit is responsible for the generation of TTX-resistant action potentials in H146 cells (Blandino et al., 1995), and whether a TTX-resistant Na_v channels contribute to migration, invasion or some other metastatic component, other than endocytic activity (Onganer et al., 2005), in SCLC cell lines. More studies are needed investigating the expression profile and role of Na_v channels in SCLC biopsy tissue.

Later analyses were also performed in non-small cell lung cancer (NSCLC) in which the $Na_v1.7$ isoform was shown to potentiate cancer cell invasion (Roger et al., 2007, Campbell et al., 2013). Although different NSCLC cell lines (H23, H460, and Calu-1) express mRNA for several Na^+ channel isoforms (Roger et al., 2007), the selective inhibition of $Na_v1.7$ activity (using TTX) or reduction of expression (by using small interfering RNA), reduced H460 cell invasion by up to 50%. On the contrary, weakly invasive A549 cells showed no evidence of functional Na_v channels (Roger et al., 2007). In addition, exogenous overexpression of the $Na_v1.7$ subunit was sufficient to promote TTX-sensitive invasion of these cells. Interestingly, $Na_v1.7$ protein expression was found to be higher in cancerous compared to normal-matched human lung tissue (Campbell et al., 2013). It is worth noting that at least in one NSCLC cell line (Calu-1), expression of a TTX-resistant Na_v channel significantly contributes to the invasion capacity of this strongly metastatic cell line. However, the molecular identity of the molecular mediator of I_{Na} has not been fully characterized. Non-quantitative PCR results suggested that mRNAs encoding the three known TTX-resistant Na_v channels ($Na_v1.5$, $Na_v1.8$ and $Na_v1.9$) may be more abundantly expressed in Calu-1 than in H23 and H460 cells (Roger et al., 2007). While kinetics and TTX-sensitivity of currents recorded in Calu-1 cells suggest $Na_V1.5$ activity, more studies are needed to properly

identify the molecular identity of the Na_V channels mediating the Na^+ current in these cells. In addition, there are currently no data correlating $Na_V\alpha$ expression in lung cancer tissue with clinical outcome.

The expression of $Na_V\beta$ in LCa has been assessed in several studies but so far it is difficult to conclude a general pattern. *SCN1B* mRNA was found to be expressed in H460, Calu-1 and A549 but not in the H23 NSCLC cell lines. It was also expressed in non-cancer NL-20 and BEAS-2B cells. *SCN2B* appeared to be weakly expressed in A549 cancer and NL-20 non-cancer cells while not expressed at all in H23, H460 and Calu-1 cancer cell lines. *SCN3B* mRNA was found to be expressed in all these cell types with the only exception of H460. *SCN4B* mRNA was expressed in cancer H23 and non-cancer NL-20 and BEAS-2B, but not in H460, Calu-1 and A549 (Roger et al., 2007). In patient samples, *SCN4B* expression levels were downregulated in lung cancer compared with normal lung tissue and preliminary immunohistochemical analyses in lung cancer tissue microarrays showed a tendency towards decreased protein expression in high-grade primary lung tumours and metastases (Bon et al., 2016). The role of the $Na_V\beta1$ protein in cell adhesion was also proposed in human non-small cell lung cancer cell lines (Campbell et al., 2013). In this study, it was shown that the highly invasive H460 cells exhibited very low expression of all $Na_V\beta$ subunit mRNAs, confirming previous results (Roger et al., 2007), whereas A549 cells expressed 8-fold higher levels of $Na_V\beta1$ mRNA. Accordingly, cell adhesion was two-fold higher in A549 cells compared to H460 cells (Campbell et al., 2013). Moreover, manipulation of $Na_V\beta1$ mRNA expression by using siRNA or cDNA targeting *SCN1B* in these two cell lines, confirmed the contribution of this subunit in the promotion of cell adhesion and reduced invasion (Campbell et al., 2013).

d- $Na_V\alpha$ and $Na_V\beta$ subunits in gastric cancer

In gastric cancer (GCa) tissue samples and in two human GCa cell lines (BGC-823 and MKN-28 cells), it was shown that the *SCN9A* gene, encoding $Na_V1.7$, is the most abundantly expressed $Na_V\alpha$ isoform (Xia et al., 2016). A systematic evaluation of 319 GCa tumour tissue samples by immunohistochemistry revealed a correlation of $Na_V1.7$ expression with poor prognosis, as well as correlation with the expression of the with NHE1 exchanger type 1 and the oncoprotein metastasis-associated in colon cancer-1 (MACC1). In addition, $Na_V1.7$ suppression resulted in reduced invasion and proliferation rates of GC cells and growth of GC xenografts in nude mice (Xia et al., 2016). In brief, results of this study indicate that $Na_V1.7$ promotes GCa progression through MACC1-mediated upregulation of NHE1.

e- $Na_V\alpha$ and $Na_V\beta$ subunits in Cervical Cancer (CeCa)

The product of the *SCN8A* gene, the $Na_V1.6$ channel, has been shown to be upregulated in cervical cancer (CeCa). In a study performed by using primary cultures derived from three different patient CeCa biopsies, the presence of functional Na_V channels has been identified and I_{Na} recorded. Primary cells from CeCa biopsies expressed mRNA for different TTX-sensitive $Na_V\alpha$ subunits: $Na_V1.1$-1.4, $Na_V1.6$ and $Na_V1.7$ (Diaz et al., 2007). Among these, only the *SCN8A* gene encoding for $Na_V1.6$ was shown to be over-expressed by about 40-fold at the mRNA level in CeCa primary cultures and biopsies in comparison with non-cancerous cervical tissue. The functional relevance of this Na_V channel was demonstrated by blocking its activity with TTX as well as with the Cn2 specific toxin, which in both cases led to a significant decrease in the invasion capacity of CeCa primary culture cells, without affecting proliferative or migratory cell behaviour (Hernandez-Plata et al., 2012). This suggested a role for $Na_V1.6$ in extracellular matrix degradation, and indeed $Na_V1.6$-mediated invasiveness of CeCa cells specifically involved MMP-2 activity along with increased expression of the NHE1 exchanger (Lopez-Charcas et al., 2018). In addition, CeCa cell lines more abundantly express the mRNA for the $Na_V1.6$ variant which has exon 18 deleted (Δ18 variant) rather than the neonatal and adult splice variants. This variant appeared to be distributed in intracellular compartments (Lopez-Charcas et al., 2018). However, the functional relevance of the Δ18 variant to the metastatic behaviour of CeCa cells remains to be elucidated. Another, interesting question regarding the expression of this Δ18 variant of $Na_V1.6$ in CeCa cells is whether it has the same function in intracellular compartments as observed in macrophages and melanoma cells in which the channel has a role in podosome formation and activity (Carrithers et al., 2009).

The role of $Na_V\beta1$ as a migration suppressor gene was demonstrated in three different CeCa cell lines (HeLa, SiHa and CaSki) in which *SCN1B* mRNA levels were around 3- to 6-fold higher than those of $Na_V\beta2$, $Na_V\beta3$ or $Na_V\beta4$. However, differences in protein levels among the four $Na_V\beta$ subunits were more discrete; $Na_V\beta1$ was again the most highly expressed in HeLa and CaSki cells, whereas in SiHa cells, protein levels for all $Na_V\beta$ were more uniform (Sanchez-Sandoval and Gomora, 2019). Previously, the same group had demonstrated that $Na_V\beta1$ mRNA levels were also slightly higher in CeCa biopsies than in non-CeCa tissue (Hernandez-Plata et al., 2012). In addition, it was demonstrated that $Na_V\beta1$ regulated SiHa cell proliferation, specifically by affecting the proportion of cells in the G0/G1 phase of

cell cycle (Sanchez-Sandoval and Gomora, 2019). Because $Na_V\beta3$ was proposed to have anti-cancer properties (Adachi et al., 2004), the effect of its expression in CeCa cells was tested. However, neither its overexpression nor its downregulation affected proliferation in CeCa cell lines, suggesting that the likely pro-apoptotic activity of $Na_V\beta3$ might not be a generalized mechanism in all cancer types or cells. In this regard, it has been suggested that the p53 protein status in CeCa cell lines is under the control of the E6 protein, the main oncogene expressed as a result of human papillomavirus (HPV) infection (the most frequent risk factor for CeCa incidence) of cervical epithelial cells. The early expression of E6 protein leads to the specific ubiquitination and degradation of p53 (Scheffner et al., 1993), therefore inactivating any pro-apoptotic effect due to the $Na_V\beta3$ expression in basal conditions. In line with this interpretation, *SCN3B* expression was increased almost 2-fold in CeCa biopsies when compared to non-cancer samples (Hernandez-Plata et al., 2012). Further studies are needed to fully understand the potential role of *SCN3B* as well as the mechanism involved in the pro-apoptotic effect in cancer cells. More recent observations in CeCa cell lines confirm the contribution of $Na_V\beta4$ to cell invasive potential as the downregulation of *SCN4B* leads to an increase in the percentage of invading cells in three CeCa cell lines (Sanchez-Sandoval and Gomora, 2019). However, a previous study indicated that mRNA levels for $Na_V\beta4$ were not significantly different between CeCa and non-CeCa biopsies tissues (Hernandez-Plata et al., 2012).

f- $Na_V\alpha$ subunits in Ovarian cancer and endometrial cancers

In ovarian cancer (OCa), the $Na_V1.5$ isoform appears to be the main $Na_V\alpha$ subunit expressed and contributing to the migration and invasion capabilities of cancer cells (Gao et al., 2010, Liu et al., 2018), however, the splicing status of $Na_V1.5$ in this carcinoma, is currently unknown.

In endometrial cancer tissues, a recent study identified the *SCN9A* gene, encoding for the $Na_V1.7$ channel, as being the most highly expressed $Na_V\alpha$ subunit. $Na_V1.7$ expression level was associated with tumour size, local lymph node metastasis, and 5-year and 10-year survival. Pharmacological inhibition using the PF-05089771 blocker selective for $Na_V1.7$ and $Na_V1.8$, induced cancer cell apoptosis and reduced cancer cell invasion (Liu et al., 2019).

g- $Na_V\beta$ in Papillary thyroid cancer

Recent results obtained in papillary thyroid cancer (PTC) show that *SCN4B* is downregulated at both RNA and protein level as compared with normal thyroid tissues (Gong et al., 2018). Importantly, by

using databases like the Gene Expression Omnibus (GEO) and the Cancer Genome Atlas (TCGA)Thyroid Cancer (THCA), the authors found that *SCN4B* expression was an independent indicator of favourable recurrence-free survival (RFS) in patients with classical PTC, further contributing to the notion of the *SCN4B* as a metastases-suppressor gene (Gong et al., 2018). So far, nothing is known about the expression of $Na_V\alpha$ subunits in PTC.

h- $Na_V\alpha$ and $Na_V\beta$ subunits in Leukaemia cells

While most results related to Na_V in cancer were obtained from solid tumours, predominantly carcinomas, there are also some indication that Na_V expression might also be dysregulated in haematological disorders such as leukaemia, in which they could bear oncogenic properties. In Jurkat leukemic T-cell lymphoblasts, original evidence showed that a small fraction of ~10% displayed I_{Na}, and mRNA encoding for $Na_V1.5$, $Na_V1.6$, and to a lesser extent $Na_V1.7$ and $Na_V1.9$, were detected (Fraser et al., 2004). I_{Na} was likely carried mostly by a TTX-resistant Na_V channel since an IC_{50} of ~1 µM was measured. Importantly, invasion was reduced by 93% when the cells were treated with 10 µM TTX (Fraser et al., 2004). However, more recent data has shown that $Na_V1.6$, $Na_V1.7$ and $Na_V1.3$ (in that order) are the most abundant Na_V isoforms in three acute lymphocytic leukaemia cell lines, including Jurkat, MOLT-4 and BALL-1 cells, as well as in peripheral blood mononuclear cells (PBMC). In this study, I_{Na} recorded from approximately 20% of MOLT-4 cells was completely abolished by 2 µM TTX, indicative of TTX-sensitive channels. The same concentration of TTX decreased the invasion of MOLT-4 and Jurkat cells by 90% (Huang et al., 2015).

Interestingly, semi-quantitative PCR results indicated the presence of both the neonatal (18N) and the Δ18 (exon 18 skipped) isoforms of $Na_V1.6$ channel in the THP-1 monocytic leukaemia cell line (Carrithers et al., 2009). Neither of these two variants form functional channels at the plasma membrane (Plummer et al., 1997). Instead, the Δ18 $Na_V1.6$ channel isoform is expressed in vesicular intracellular compartments and crucially contributes in the control of podosome and invadopodia formation (Carrithers et al., 2009). In addition, *SCN5A* ($Na_V1.5$) is expressed in the late endosome, rather than at the plasma membrane of the THP-1 cells. The intracellular $Na_V1.5$ channel was shown to enhance endosomal acidification and phagocytosis (Carrithers et al., 2007), Ca^{2+} signalling and phenotypic differentiation in human macrophages (Carrithers et al., 2011). The same group later demonstrated that *SCN5A* was expressed as a new splice variant lacking exon 25, resulting in a deletion of 18 amino acids in domain III (Rahgozar et al., 2013), generating non-selective outward currents and small inward currents in a heterologous expression system (Jones et al., 2014).

i- $Na_V\alpha$ in Ewing Sarcoma

The Ewing Sarcoma (ES) is the second most common primary malignant bone tumour in children and adolescents, following osteosarcoma (Choi et al., 2014). RING1B, a member of the Polycomb family of epigenetic regulators, is highly expressed in primary ES tumours. Depletion of RING1B with shRNA in ES cells enriched the expression of genes involved in haematological development, without affecting cellular differentiation (Hernandez-Munoz et al., 2016). Importantly, in ES cells, RING1B directly binds to the promoter of *SCN8A* and its depletion results in enhanced $Na_V1.6$ expression and function. In addition, the migratory speed of RING1B-depleted ES cells was attenuated, suggesting an inverse correlation between *SCN8A* expression and the migration capabilities of ES cells. Finally, reduced $Na_V1.6$ function appeared to protect ES cells from apoptosis by a mechanism that maintains low NF-κB levels (Hernandez-Munoz et al., 2016). These findings revealed striking differences in the participation of *SCN8A* and its product, the $Na_V1.6$ channel, in sarcomas compared to carcinomas and leukaemia. Indeed, $Na_V1.6$ appeared to have anti-cancer properties in ES while it has pro-invasive functions in carcinomas and leukaemia. Therefore, further studies are needed to fully understand the function of *SCN8A* across different types of cancer.

II- Conclusions on the roles of $Na_V\alpha$ and $Na_V\beta$ subunits in cancers

α-Pore-forming $Na_V\alpha$ subunits

As previously indicated, the three main $Na_V\alpha$-encoding genes found to be upregulated in cancers are *SCN5A, SCN8A,* and *SCN9A,* which encode $Na_V1.5$, $Na_V1.6$ and $Na_V1.7$ respectively. On the other hand, recent reports showed the downregulation of *SCN8A* and *SCN9A* genes in some cases. The molecular determinants explaining why these specific isoforms are overexpressed in cancers are not known and might be tissue-specific. However, it is tempting to consider the deregulation in tumours of transcription factors which normally restrict the expression of a suite of genes associated with specific tissue functioning in adult tissues, such as the repressor element silencing transcription factor (REST) which restrict the expression of $Na_V\alpha$ channels in excitable cells (Bruce et al., 2004, Chong et al., 1995) or other epigenetic regulations such as histone acetylation/deacetylation (performed by Histone Acetylases HAT and Histones Deacetylases HDAC, respectively), DNA or histone methylation. Indeed, it was recently proposed that REST and HDAC2 play important role as epigenetic regulators and their inhibition in MCF-7 breast cancer cells enhanced the expression of $Na_V1.5$ and promoted invasive capacities (Kamarulzaman et al., 2017). Nevertheless, it is interesting to notice that, when specifically

studied in cancer cells, several neonatal splice variants of channels have been identified (Fraser et al., 2005, Lopez-Charcas et al., 2018, Baptista-Hon et al., 2014, Carrithers et al., 2009), thus supporting the common hypothesis of the re-expression of developmental genes in cancers.

These channels are present at the plasma membrane of cancer cells where sodium currents have been recorded. With the exception of $Na_V1.6$ in Ewing sarcoma cells (Hernandez-Munoz et al., 2016), all $Na_V\alpha$ isoforms have been shown to bear oncogenic properties, promoting cancer cell invasion *in vitro*, as well as other behaviours associated with metastasis, such as the acquisition of elongated and mesenchymal-like phenotypes, directed migration, proliferation, regulation of endocytosis, control of intracellular and perimembrane pH and extracellular matrix degradation (Yang et al., 2012, Hernandez-Plata et al., 2012, Roger et al., 2003, Grimes et al., 1995, Fraser et al., 2005, Gillet et al., 2009, Mycielska et al., 2003, Djamgoz et al., 2001, Brisson et al., 2013). In addition, $Na_V\alpha$ subunits promote tumour growth, invasion, and metastasis in *in vivo* rodent models (Nelson et al., 2015b, Driffort et al., 2014, Batcioglu et al., 2012, Yildirim et al., 2012). Comparative studies performed in different cancer types indicate the involvement of these $Na_V\alpha$ isoforms in similar functional properties, arguing for isoform-independent signalling pathways. The activity of the channels at the plasma membrane appears to be critical. Indeed, Navα subunits are functionally active in cancer cell lines and primary tumour cells cultured *in vitro*, as well as in murine tumour xenograft tissue slices *in vivo* (Fraser et al., 2005, Roger et al., 2003, Hernandez-Plata et al., 2012, Nelson et al., 2015b) and their inhibition, using different drugs and small molecules such as TTX, ranolazine, phenytoin, Cn2 or PF-05089771, inhibit invasion (Nelson et al., 2015a, Nelson et al., 2015b, Driffort et al., 2014, Batcioglu et al., 2012, Yildirim et al., 2012, Lopez-Charcas et al., 2018, Roger et al., 2003, Roger et al., 2007, Liu et al., 2019).

Importantly, the membrane potential (V_m) of cancer cells is typically relatively depolarised compared to terminally differentiated non-cancer cells (Yang and Brackenbury, 2013). At this range of Vm, $Na_V\alpha$ channels would be expected to be predominantly in the inactivated state. However, in cancer cells the V_m is generally between -40 and -30 mV and is situated in a window of voltage which provides a small non-inactivating persistent Na^+ current flowing into the cell locally increasing intracellular Na^+ concentration (Yang et al., 2012, Roger et al., 2003, Campbell et al., 2013). Recently a Na^+-dependent intracellular signalling pathway, involving Salt-inducible kinase 1, has been proposed to account for pro-invasive effects of $Na_V\alpha$ (Gradek et al., 2019).

The main role attributed to Na^+ is to serve as a mere mediator of the membrane potential, in excitable as well as in non-excitable cells. It is also characterized to support ions (among which Na^+/K^+, Na^+/Ca^{2+}, $Na^+/K^+/Cl^-$, Na^+/HCO_3^-) exchanges and nutrients / metabolites transports across membranes

(Na^+/glucose for example). The role of second messenger is mostly attributed to the Ca^{2+} ion, for which a lot of specific probes and tools have been developed over the last twenty years. In contrast, no direct and specific biological sensors for Na^+ have been identified, and tools to study Na^+ evolution still lack sensitivity or dynamics. Yet, there is some evidence suggesting that Na^+ could act as a second messenger *per se* and might regulate several important signalling pathways in normal cells. Indeed, recent data support a direct role of Na^+ in controlling kinases activity (Jaitovich and Bertorello, 2010), membrane fluidity and protein diffusion through an interaction with phospholipids (Hernansanz-Agustin et al., 2020) or to induce inflammatory stress (Amara et al., 2016). Therefore, this raises the possibility that Na^+ could also serve as a second messenger in cancer cells to activate signalling pathways promoting aggressiveness. To further support this hypothesis, it is worth mentioning that early studies questioned the involvement of intracellular Na^+ content and the consequences on malignant cell proliferation, invasive capacities and the development of metastases (Cone, 1974). Indeed, much higher Na^+ concentrations have been recorded in tumour cells, as compared to non-cancer cells by energy-dispersive X-ray microanalyses (Cameron et al., 1980) as well as by 23Na-magnetic resonance imaging (Ouwerkerk et al., 2007, Jacobs et al., 2004, Zaric et al., 2016) and was proposed to serve as an indicator of malignancy.

The inward Na^+ current may also further depolarise the V_m which might also participate in promoting migration. In support of this hypothesis, it was demonstrated in breast cancer cells that $Na_V1.5$ sustained V_m depolarization which activated the RhoGTPase Rac1, subsequently inducing cytoskeletal reorganization and cellular migration (Yang et al., 2020) (Figure 2). While there is clear evidence that $Na_V\alpha$ channels expressed at the plasma membrane are critical in the acquisition of oncogenic properties, the discovery of splice variants with expression restricted to intracellular compartments, such as endosomes, phagosomes or lysosomes, (Lopez-Charcas et al., 2018, Carrithers et al., 2009) suggests a more complex role.

b- (More than) Auxiliary $Na_V\beta$ subunits

The main $Na_V\beta$-encoding gene found to be upregulated *in cancers* is *SCN1B,* encoding for the $Na_V\beta1$ subunit (Diss et al., 2008, Nelson et al., 2014, Bon et al., 2016, Sanchez-Sandoval and Gomora, 2019). On the other hand, there are multiple reports showing the downregulation of *SCN4B* ($Na_V\beta4$) (Hernandez-Plata et al., 2012, Bon et al., 2016, Diss et al., 2008, Gong et al., 2018, Sanchez-Sandoval and Gomora, 2019) and of *SCN3B* ($Na_V\beta3$) in some instances (Bon et al., 2016, Adachi et al., 2004).

The $Na_V\beta1$ subunit has been shown to increase cancer proliferation, cell adhesion, increase neurite-like process outgrowth formation, and promote cancer cell invasion, whilst slowing migration *in vitro* (Nelson et al., 2014, Chioni et al., 2009, Bon et al., 2016, Sanchez-Sandoval and Gomora, 2019). *In vivo*, $Na_V\beta1$ overexpression increases angiogenesis and reduces apoptosis, thus increasing tumour growth and metastasis (Nelson et al., 2014). Taken together, these results are in favour of a pro-cancerous role of $Na_V\beta1$ and the effects appear to be dependent in part on the regulation of the $Na_V\alpha$ pore-forming subunit and as well as being dependent on the extracellular CAM motif (Nelson et al., 2014). $Na_V\beta2$ expression in prostate cancer cells also increases process extension, adhesion, invasion and migration *in vitro*, but reduces tumour take *in vivo* (Jansson et al., 2014, Jansson et al., 2012). Conversely, $Na_V\beta3$ and $Na_V\beta4$ may function as tumour suppressors. The *SCN3B* gene contains p53 response elements and $Na_V\beta3$ suppresses colony formation and promotes chemotherapy-induced apoptosis in a p53-dependent manner (Adachi et al., 2004). Na_V β4 expression is downregulated in breast, colorectal, lung, cervical, prostate tumours and papillary thyroid cancer compared with normal tissue (Hernandez-Plata et al., 2012, Bon et al., 2016, Diss et al., 2008, Gong et al., 2018, Sanchez-Sandoval and Gomora, 2019). In addition, $Na_V\beta4$ functions as a tumour and metastasis suppressor gene *in vivo* (Bon et al., 2016). This tumour-suppressing function occurs via β4mediated control of RhoA GTPase activation (Bon et al., 2016) (Figure 2).

III- $Na_V\alpha$ as anticancer targets for repurposed drugs and new small inhibitory molecules

$Na_V\alpha$ are attractive drug targets because of the broad therapeutic potential of their blockers. Considering the fact that $Na_V\alpha$ are expressed in metastatic cells in various tumours, significant effort has been made to develop $Na_V\alpha$ blockers as potential drugs for cancer treatment. This section of the review focuses on such efforts that took place in the past 10 years. These efforts for blocker development can broadly be classified in to two sections. 1) Repurposing drugs that are FDA-approved for other clinical uses (local and general anaesthetics, antiepileptic and anticonvulsant, antiarrhythmic drugs). 2) Rational design and development of novel $Na_V\alpha$ blockers for cancer treatment.

α- Repurposing of FDA-approved $Na_V\alpha$ blockers

There are numerous existing $Na_V\alpha$ inhibitors licensed for clinical use. In several cases $Na_V\alpha$ inhibition is considered an off-target effect of these drugs. For example, tricyclic antidepressants, including amitriptyline, inhibit the serotonin transporter, but also inhibit several neurotransmitter receptors and voltage-activated ion channels including $Na_V\alpha$. In other cases $Na_V\alpha$ inhibition is

considered the primary mechanism of the drug's intended therapeutic effect. This is true for several anti-seizure medications (e.g. phenytoin and carbamazepine) and all of the local anaesthetics, although these too have additional off-target actions. Regardless of whether it is a primary or secondary effect of a licensed medication, $Na_V\alpha$ inhibition might be beneficial in patients with cancers associated with $Na_V\alpha$ expression. This raises the intriguing possibility that approved $Na_V\alpha$ inhibiting drugs might be repurposed to treat cancer.

Benefits of repurposing approved medications include prior knowledge of their mechanisms of action and the availability of toxicology and safety data, thereby avoiding the need for drug discovery and early phase clinical trials. Drawbacks include limited potential for developing intellectual property, leading to a lack of both funding potential and industry involvement (Pushpakom et al., 2019). Nevertheless, despite the potential drawbacks, there are some notable successes and a well-trodden pathway to repurposing is through the use of electronic health records to link prescribing data to potentially beneficial health outcomes. A good example of the impact of this type of retrospective clinical analysis is the identification of an association of aspirin-use with reduced risk of colon cancer (Dube et al., 2007). Similar approaches are being used in studies exploring a possible relationship between $Na_V\alpha$ inhibitors and outcomes in cancer patients.

Anti-seizure and class 1 antiarrhythmic $Na_V\alpha$ inhibitors

A recent study, using retrospective clinical analysis to explore the possibility of a beneficial effect of Na_V inhibiting medications, examined several class 1 antiarrhythmic and antiseizure medications in patients with breast, bowel or prostate cancer (Fairhurst et al., 2015). The combined analysis revealed that these medications (including Class I antiarrhythmic drugs, lamotrigine, carbamazepine phenytoin, and valproate) were collectively associated with decreased median time to death compared to the control patient group, with significantly increased mortality in the drug group. This study clearly does not support the idea of repurposing antiseizure $Na_V\alpha$ inhibitors in the treatment of breast, bowel or prostate these cancers. However, as the authors pointed out, the causes of death were not available in the large primary care dataset and co-morbidities were among the likely confounding factors. In many cases, patients treated with $Na_V\alpha$ inhibiting drugs will be suffering from life-threatening disorders such as epilepsy and it is difficult to completely accommodate this confound in retrospective analyses.

Analgesic $Na_V\alpha$ inhibitors

There has been considerable recent interest in the idea that anaesthetics and analgesics used during surgical tumour excision might influence subsequent cancer recurrence. Surgery can cause the release of tumour cells into the circulation and the number of postsurgical circulating cancer cells is known to be a negative prognostic indicator of disease-free survival (Yu et al., 2018). The perioperative period, *i.e.* immediately before, during and after surgery, may therefore be an opportune time for interventions that inhibit the potential for metastatic invasion. A variety of drugs are typically administered during surgery including general anaesthetics, analgesics, anti-muscarinic and neuromuscular blockers. Some of these, such as inhalational general anaesthetics, may have the potential to worsen outcomes by suppressing the immune response (Stollings et al., 2016). By contrast, local anaesthetics may provide more favourable outcomes. Local anaesthetics are often administered regionally to provide blockade of afferent nociceptive fibres entering the spinal cord. Several retrospective clinical studies suggest that regional analgesia during breast and prostate cancer surgery increases disease free survival (Forget et al., 2019). The use of regional anaesthesia diminishes or abolishes the need for general anaesthetic during surgery. It was therefore initially hypothesised that the general anaesthetic sparing effect of regional analgesia with local anaesthetics accounts for the apparent beneficial effect (Sessler et al., 2008). However, a recent large prospective multicentre trial comparing outcomes after breast cancer surgery under inhalational anaesthesia with or without paravertebral analgesia by ropivacaine or levobupivacaine revealed no difference in disease free survival (Sessler et al., 2019).

Most of the ongoing clinical trials exploring the impact of anaesthetic technique on cancer outcomes are predicated on the idea that the potential benefit of local anaesthetics is conferred indirectly through their inhalational anaesthetic sparing effect. However, it is possible that local anaesthetics such as lidocaine, ropivacaine and levobupivacaine provide a direct beneficial effect through $Na_V\alpha$ inhibition (Baptista-Hon et al., 2014, Elajnaf et al., 2018). If this is the case then a more direct approach for administering these drugs directly onto the tumour may prove to be beneficial. Lidocaine, in addition to being a local anaesthetic, is also used intravenously as a class 1b antiarrhythmic agent and a circulating analgesic. Ongoing clinical trials will test whether lidocaine delivered directly onto breast tumours prior to excision, or intravenously during the perioperative period for colon cancer surgery will prolong postoperative disease-free survival (NCT01916317, R.A Badwe, 2013; NCT02786329, D. Ionescu, 2016). We await the outcome of these trials with interest and note that there are several other approved $Na_V\alpha$ inhibiting drugs that should be examined in retrospective clinical studies for potential beneficial effects in cancer outcomes.

b- Rational design of small molecule $Na_V\alpha$ blockers

Rational designing of $Na_V\alpha$ inhibitors has been difficult since detailed structural information of drug binding sites for this integral membrane protein were lacking until very recently. Therefore, early effort for the discovery of $Na_V\alpha$ blockers mainly relied on strategies such as ligand-based drug design, natural product based drug design, *in silico* screening and similarity searches. However, recent reports on the structures of human and bacterial $Na_V\alpha$ and bound ligands shed light on their binding site (Cervenka et al., 2018, Nguyen et al., 2019, Pan et al., 2018, Payandeh et al., 2011, Shen et al., 2017, Shen et al., 2018, Shen et al., 2019). These reports will certainly aid in the structure-based design and discovery of $Na_V\alpha$ blockers. A summary of the available reports on the identification and evaluation of $Na_V\alpha$ blockers for potential use in cancer therapy follows.

One such effort to identify $Na_V\alpha$ blockers used a pyrrole-imidazole marine alkaloid, clathrodin (Hodnik et al., 2013). Clathrodin was originally isolated from the *Agelas clathrodes* sponge. Several conformationally restricted analogues of clathrodin containing a 4,5,6,7-tetrahydrobenzo [d] thiazol2-amine moiety are blockers of $Na_V1.3$, $Na_V1.4$, $Na_V1.5$ and $Na_V1.7$ channels. These compounds display state-dependent inhibitory activity of these channels at low micromolar concentrations. The most active compound (4e, Figure 3) identified from this study represents a novel selective blocker of $Na_V1.4$ channel with an IC_{50} value of 8 µM. The use of clathrodin analogues as a template for ligand based virtual screening of commercially available ZINC library of compounds using ROCS software, identified two potent lead compounds 2 and 16 (Tomasic et al., 2013) (Figure 3). These blocked I_{Na} produced by $Na_V1.7$ with IC_{50} values of 7 µM and 9 µM, respectively.

Plant derived polyphenolic natural products have also been reported as $Na_V\alpha$ inhibitors. For example, the plant phenolic, resveratrol, (Figure 3) found at high concentrations in red grapes inhibits $Na_V\alpha$ with an IC_{50} value of 50 µM (Fraser et al., 2014). Resveratrol also suppresses lateral cell motility by up to 25%, transverse cell motility by 31%; and cell invasion by 37%, without affecting cellular proliferation or cell viability of MAT-LyLu cells. Another similar phenolic is caffeic acid phenethyl ester (CAPE, Figure 3) isolated from honeybee propolis. CAPE blocks Na_V activity in several invasive cancer cell lines including breast (MDA-MB-231 and MDA-MB-468), colon (SW620) and non-small cell lung cancer (H460). Motility and invasion of MDA-MB-231 cells were reduced by up to 14% and 51%, respectively by CAPE at 1 µM without affecting cell proliferative activity (Fraser et al., 2016).

Shaheen et al. used an *in silico* approach to identify $Na_V\alpha$ blockers with superior pharmacological profile compared to phenytoin (PHT) and carbamazepine (CBZ) in 2015 (Shaheen et al., 2015). They

conducted a similarity search in the PubChem database with PHT and CBZ as query molecules using the Tanimoto based similarity search. The search was further refined by docking of these molecules into the binding site of the homology model of *SCN1A* using MolDock program. This study identified high affinity compounds similar to PHT and CBZ. The lead compounds were further evaluated for toxicity profiles and biological activity. Two of the best compounds identified by this study, NSC403438 and AGN-PC-0BPCBP (Figure 3) demonstrated better binding affinity to $Na_V\alpha$ compared to PHT and CBZ, with NSC403438 being a superior inhibitor of I_{Na} with lower toxicity, better IC_{50} value and optimal bioactivity.

$Na_V\alpha$ inhibitors were also derived from the natural product, crambescin (Nakazaki et al., 2016). Enantiomerically pure crambescin A, B and C carboxylic acid derivatives were synthesized and evaluated for their ability to block $Na_V\alpha$. Structure activity relationship studies revealed that the natural enantiomer of crambescin B, carboxylic acid, (Figure 3) is the most active compound with activity comparable to TTX. The cyclic guanidinium moiety present in this molecule is indispensable for its activity.

In 2018, Dutta et al. utilized a highly predictive, comprehensive 3D-QSAR model for the design of $Na_V\alpha$ blockers (Dutta et al., 2018). The $Na_V\alpha$ binding data (IC_{50}) for 67 compounds were used to train a comprehensive CoMFA model, which effectively covered 3D space and spanned over 4 orders of magnitude in biological activity. Potency predictions by this model have been highly accurate for the more than 30 compounds that were synthesized and evaluated. Five compounds shown or predicted to have low nanomolar $Na_V\alpha$ binding were further evaluated for the inhibition of $hNa_V1.5e$ currents in individual breast cancer MDA-MB-231 cells. Of these, two lead compounds, 1 and 4 (Figure 3) were found to be most effective in whole cell patch-clamp studies and showed significant invasion inhibitory activities at concentrations as low as 1 µM without affecting cell viability.

Boezio et al. reported several sulfonamides with highly selective $Na_V1.7$ blockade activity (Boezio et al., 2018). This novel series of blockers contained a triazole sulfone which served as a bioisostere for the acyl sulfonamide group. This work resulted in the discovery of a series of potent $Na_V1.7$ blockers with selectivity over $Na_V1.5$ and favourable pharmacokinetic properties in rodents. An example of such a blocker is compound 35 (Figure 3). In the same year, Yapa et al. reported a known inhibitor of TRPM8, N-(3-aminopropyl)-2-{[(3-methylphenyl) methyl]oxy}-N-(2-thienyl methyl)benzamide (AMTB, Figure 3) as a $Na_V\alpha$ blocker in breast cancer MDA-MB-231 cells (Yapa et al., 2018). AMTB decreased viable cell number in MDA-MB-231 and SK-BR-3 breast cancer cell lines and also reduced the migration of MDA-MB-231 cells. These studies provided the evidence that these effects are not related to TRPM8 inhibition, but rather caused by the $Na_V\alpha$ blockade caused by AMTB. Gumushan Aktas et al.

investigated the potential effects of a natural flavanone, naringenin (Figure 3) on the motility of MAT-LyLu cells in 2018 (Gumushan Aktas and Akgun, 2018). This study revealed that naringenin inhibited cell proliferation at higher concentrations (75 µM), whereas it decreased the movement of MAT-LyLu cells at low concentrations (5 µM and 10 µM). Moreover, naringenin inhibited cell motility by reducing the expression of the *SCN9A* gene at the mRNA level. In conclusion, naringenin was found to have direct or indirect blocking activity on the *SCN9A* encoded channel. Most recently, in 2019, Wang et al. has reported $Na_V1.7$ channel blockers using a comparative molecular field analysis (CoMFA) model for the binding of ligand to $Na_V\alpha$ was generated based on diverse set of compounds. No channel current blockade data was presented in the paper.

However, there was an extensive anticancer evaluation of the identified lead compounds S0154 and S0161 (Wang et al., 2019). Both showed anticancer and anti-metastatic effects against PC3 prostate cancer cells and significantly inhibited cell viability, with IC_{50} values in the range of 5-26 µM. Both these compounds inhibited the expression of $Na_V\alpha$, increased the intracellular level of Na^+, and caused cell cycle arrest in G2/M phase. The compounds also inhibited the invasion of PC3 cells. Furthermore, S0161 inhibited the PC3 tumour growth of by about 51% in an in vivo xenograft model (Wang et al., 2019).

In conclusion, there have been major advances in $Na_V\alpha$ targeted drug discovery over the last decade. Specific $Na_V\alpha$ isoforms have been implicated in the metastasis development of a variety of tumours raising the possibility of developing tumour selective drugs. Recent advances in the discovery of high-resolution crystal and cryo-EM structures of $Na_V\alpha$ should further advance the field with structure-based drug design efforts.

IV- $Na_V\alpha$ as targets for nutritional management of cancers

Cancer is a metabolic disease which depends on bioenergetic parameters (Penkert et al., 2016). Cancer progression is generally associated with the survival of cancer cells under conditions of low oxygen levels and nutrient deprivation, and relies on metabolic adaptations (Dumas et al., 2017, Hanahan and Weinberg, 2011). These metabolic adaptations allow cancer cells to survive the pressure of environmental conditions and to fulfil the high energy demands associated with their high anabolic activity (Payen et al., 2016, Porporato et al., 2016). Also, this metabolic switch towards an aerobic glycolysis brings selective advantages by promoting invasive activities and metastatic properties (Brisson et al., 2012, Webb et al., 2011). Furthermore, the metabolic reprogramming does not only concern tumour cells, but also multiple cell types and organs of the host, thus leading to an overall

deregulation of the energetic balance in patients, called tumour cachexia (Fearon et al., 2012). This devastating syndrome, initially triggered by the release of soluble tumour factors and the participation of a systemic inflammation, is characterized by anorexia, the loss of skeletal muscle mass, in some cases with the loss of adipose tissue mass, and a general weakening of patients, impeding their quality of life and decreasing the tolerance to antineoplastic therapies (Fonseca et al., 2020, Biswas and Acharyya, 2020). In this context, bringing a nutritional support to patients is required to allow holding the most efficient possible treatment. Nutritional interventions are mostly aimed at preventing the wasting of body compartments in patients, but could also be a source of active anti-cancer molecules. Furthermore, diet represents a controllable component of the environment and brings promising strategies to increase treatment efficacy in combination with conventional chemotherapeutics. Some dietary compounds have also been shown to decrease the risk of carcinoma development and may prolong the survival of patients (Dumas et al., 2017).

As pointed out in the previous section, several natural compounds in the diet, such as resveratrol and caffeic acid phenethyl esters, are effective at inhibiting $Na_V\alpha$ subunits (Fraser et al., 2016, Fraser et al., 2014). Dietary lipids have also been proposed to inhibit $Na_V\alpha$ and to modulate cancer progression. Indeed, dietary lipids incorporate into cellular membrane and alter $Na_V\alpha$ or their pharmacology (Agwa et al., 2018, D'Avanzo, 2016). Among dietary lipids, long chain n-3 polyunsaturated fatty acids (n-3 PUFA) have been described in epidemiological studies to delay or prevent the appearance of breast cancer (Rose et al., 1996, Bougnoux et al., 2010). From both *in vivo* and *in vitro* studies, n-3 PUFA have been reported to induce multiple anti-tumour effects and their dietary consumption was associated with a lower risk of cancers, such as breast or colorectal cancers (Bougnoux, 1999, Bougnoux et al., 2010, Eltweri et al., 2016). Even though n-3 PUFA were suggested to prevent prostate cancer (Moreel et al., 2014, Li et al., 2017), their beneficial effect remains to be demonstrated through more intervention trials or observational studies (Aucoin et al., 2016). A pilot study showed that fatty acid composition of breast adipose tissue differed according to breast cancer focality : low levels of the two long chain n-3 PUFA docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3) were associated with tumour multifocality, which is considered a marker of cancer aggressiveness (Ouldamer et al., 2016a). These results could indicate that differences in adipose tissue concentration, a surrogate of past dietary uptake, may contribute to mechanisms influencing cancer progression.

Long-chain n-3 PUFA have been proposed to increase tumour sensitivity to chemotherapeutic agents with no sensitization of normal tissues and no additional side effect (Bougnoux et al., 2010). As such, DHA and EPA have generated intense interest due to their ability to reduce resistance to

anthracyclines, taxanes or radiotherapy in mammary tumour models (Bougnoux et al., 2010, Hajjaji et al., 2012, Hajjaji et al., 2011, Ouldamer et al., 2016b).

N-3 PUFA have also been shown to have anti-invasive and anti-metastatic properties (Blanckaert et al., 2010, Gillet et al., 2011, Rose et al., 1997, Rose et al., 1994, Bougnoux et al., 2010). On the other hand, they are capable of modulating the activity of NHE exchangers (Besson et al., 1996, Lacroix et al., 2008) and ion channels (Tillman and Cascio, 2003, Jude et al., 2003). Interestingly, in expression systems and in native rat cardiomyocytes, the activity of $Na_V1.5$ was initially found to be inhibited by n-3 PUFA (Kang and Leaf, 1996, Kang et al., 1997), and in initial studies have proposed that these effects could be mediated by a directly binding of n-3 PUFA to specific residues of the channel (Xiao et al., 2001, Xiao et al., 1998). Therefore, n-3 PUFA could also exert their beneficial effects on cancers through a reduction of $Na_V1.5$ (Pignier et al., 2007, Gillet et al., 2011). However, contrasting results were obtained in human breast cancer cells in which I_{Na} was not inhibited by acute applications of n-3 PUFA, even at high concentrations (30-50 µM) (Wannous et al., 2015) at which they also have anti-proliferative effects (Barascu et al., 2005). This discrepancy might be due to the fact that cancer cells mostly express the $hNa_V1.5e$ neonatal splice variant (Fraser et al., 2005). However, growing breast cancer cells in the presence of low doses of DHA (0.5 to 10 µM) reduced *SCN5A* gene expression and levels of $Na_V1.5$ proteins and I_{Na} (Wannous et al., 2015, Isbilen et al., 2006). This inhibition of *SCN5A* expression was mediated by the lipid-sensitive nuclear receptor Peroxisome Proliferator Activated Receptor –β (PPAR-β). Correlatively, the inhibition of $Na_V1.5$ activity was also responsible for a reduced activity of the downstream protagonist NHE-1, thus decreasing H^+ efflux, preventing extracellular matrix degradation proteolytic activity and inhibiting breast cancer cell invasiveness (Wannous et al., 2015). A recent report also demonstrated the efficacy of EPA to reduce migration and proliferation of ovarian cancer cells by inhibiting $Na_V1.5$ (Liu et al., 2018).

Such regulations concerning other $Na_V\alpha$ involved in cancer properties, *i.e.* $Na_V1.6$ and $Na_V1.7$, should be investigated. It is also of interest to note that n-3 PUFA, through the activation of PPAR-γ, have been shown to down-regulate the expression of NHE-1 and reduce cancer colony growth (Kumar et al., 2009). Furthermore, incorporation of n-3 PUFA into phospholipids induces changes in the physico-chemical properties of cell membranes (Yaqoob and Shaikh, 2010) which in turn affects NHE1 activity (Dendele et al., 2014).

Further to these effects on Na_V channels and downstream signalling pathways, it should be mentioned that n-3 PUFA might exert a multiplicity of actions by interfering with several signalling pathways, some of them being beneficial to the prevention or treatment of cancer. As such, a lack of specificity

is not obligatorily detrimental and pleiotropic effects might increase the efficacy of the anticancer treatment.

N-3 PUFA supplementations were proposed to have beneficial effects in reducing mammary tumour growth, by slowing down cancer cell proliferation (Barascu et al., 2005). N-3 PUFA treatment was demonstrated to inhibit cyclin B1 and the expression of the cell division cycle 25C phosphatase, which dephosphorylates cyclin-dependent kinase 1 (Barascu et al., 2005). In addition, the nuclear receptor PPARβ appeared to regulate the DHA-related inhibition of MDA-MB-231 and MCF-7 cells proliferation. This allowed identifying PPARβ as an important protagonist in the inhibition of breast cancer cell proliferation and mammary tumour growth under DHA-enriched diet (Wannous et al., 2013). N-3 PUFA have been proposed to regulate autophagy in cancer cells, and as such could be involved in both survival and apoptosis, depending on the carcinogenetic phase and on the treatment context (Ferro et al., 2020). DHA was found to induce apoptosis in cancer cells (Jing et al., 2011). DHA-induced autophagy was associated with p53 loss, with the activation of AMPK and with the decrease in the activity of mTOR. Autophagy inhibition suppressed apoptosis, and autophagy induction further enhanced apoptosis in response to DHA treatment (Jing et al., 2011). There is evidence that n-3 PUFA may inhibit the expression of EMT markers and reduce associated invasive properties in cancer cells (D'Eliseo et al., 2016, Yin et al., 2016). Altogether, these effects could bring beneficial values to delay the appearance/diagnosis of a primary tumour, and as such be of interest in primary prevention.

N-3 PUFA, which are highly peroxidizable, were also proposed to improve the efficacy of anticancer treatments by amplifying oxidative stress generated by anthracyclines or radiotherapy (Bougnoux et al., 2009). The acquisition of resistance to chemotherapeutic agents represents an important limitation in cancer. Resistance to taxanes has been proposed to depend on the induction of signalling pathways such as PI3K/Akt and ERK1/2, which promote survival and cell growth in human cancer cells. In docetaxel-treated MDA-MB-231 cells, phosphorylated-ERK1/2 levels were increased by 60% in both membrane and nuclear compartments, compared to untreated cells and ERK1/2 activation depended on PKCε and PKCδ activation. In comparison, in cells treated with DHA, docetaxel was unable to increase PKCε and PKCδ levels, thus resulting in the reduction of ERK1/2 phosphorylation and the increase in docetaxel efficacy (Chauvin et al., 2016). In addition to these effects, n-3 PUFA were proposed to increase the efficacy of the chemotherapeutic treatment by remodelling the tumour vascular network, thereby improving the delivery of anticancer drugs within the tumour (Kornfeld et al., 2012). These results support the hypothesis that n-3 PUFA, which do not induce any toxic effect, could be used as an adjuvant to cancer therapy.

V- Conclusions and perspective for the treatment of cancers

There is now clear evidence that the abnormal expression of Na_V subunits occurs during carcinogenesis is associated with cancer progression towards metastatic states. Several different $Na_V\alpha$ play a role, including $Na_V1.5$, $Na_V1.6$ and $Na_V1.7$, but the intracellular signaling pathways they regulate appear to be the same or very similar in all studied cancer types, leading to the induction of invasive properties. The activity of such channels at the plasma membrane, and consequent I_{Na}, appear critical. Therefore, the inhibition of $Na_V\alpha$ in cancer cells represents a new anti-cancer strategy which could be achieved in several ways alone or in combination: repurposing existing $Na_V\alpha$– inhibitory drugs, developing new small inhibitory molecules, and through dietary interventions. As shown above, $Na_V\beta$ subunits also have important roles in cancer cell biology and in cancer progression, acting both as auxiliary subunits of $Na_V\alpha$ and as CAMs. However, they do not exert a recordable activity *per se* and their direct "inhibition" or "activation" would be challenging. Therefore, dietary strategies aiming at controlling their expression, *i.e.* reducing the expression of *SCN1B* and *SCN2B*, while maintaining the expression of *SCN3B* and *SCN4B*, might represent powerful strategies. For this purpose, future studies aiming at unravelling transcriptional and epigenetic regulators will be of high interest.

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Declaration of interests

Author declare no competing interest.

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Figure legends

Figure 1: Expression of $Na_V\alpha$ in carcinoma and role in invadopodial activity and invasion of extracellular matrices.

Progression of precancerous into cancer cells is illustrated in the context in the malignant transformation of colon epithelium. Transformed cells have lost cell polarity, replication control, cell-cell adherent junctions and they acquired a mesenchymal pro-invasive phenotype. Migrating cancer cells develop a specialized actin-based membrane protrusions called "invadopodia" that facilitate cell invasion by providing a coupling of focal extracellular matrix (ECM) degradation together with a directional cell movement. Na_V channels are expressed in invadopodial structures, co-localizing with the Na^+/H^+ exchanger type 1 (NHE1). Activity of Na_V channels enhances the extrusion of protons by NHE1 and therefore the acidification of the peri-invadopodial microenvironment, thus favouring both secretion and activity of ECM proteases such as cysteine cathepsins and matrix metalloproteinases (MMPs). Cancer cell resting potential (V_m) is around -40 mV, in a window of voltage of Na_V channels (overlap between activation and steady-state inactivation curves) in which a small proportion of channels are activated but non-inactivated, thus generating a small but continuous Na^+ influx through a so-called "window sodium current". Na_V channels are also proposed to increase the intracellular levels of Ca^{2+} ions by the functioning of Na^+/Ca^{2+} exchanger (NCX) in a "reverse mode". Thus, the increase in the intracellular concentration of Na^+, and Ca^{2+}, sustains SRC kinase activity leading to the polymerization of acting filaments and the formation of invadopodial structure.

Figure 2: Participation of $Na_V\alpha$ and/or $Na_V\beta$ in pro-metastatic signalling pathways.

$Na_V\alpha$ subunit over-expression and activity in cancer cells trigger biochemical or an electro-biochemical cascades leading to the acquisition of a pro-invasive cell phenotype. Na_V is co-localized with NHE1 in caveolin-1 (Cav-1)-containing lipid rafts and promotes the efflux of protons. Na_V activity can be further stimulated by the use of pharmacological activators such as veratridine (inhibitor of the inactivation phase). Activity of $Na_V\alpha$ subunits leads to a cAMP-independent activation of protein kinase A (PKA) that activates the cytosolic small GTPase Ras-related protein 1 (Rap1A/B) and the extracellular signal-regulated kinases (ERK1/2). The transcription factor (TF) metastasis-associated in colon cancer 1 (MACC1) is activated by the p38/NF-κβ signalling while the TFs c-jun, ELK1 and ETS1 are activated by ERK1/2 and the activation of the zinc finger protein SNAI1 through a $Na_V\alpha$dependent mechanism regulating the expression of genes associated with cytoskeleton reorganization, cell motility, extracellular matrix degradation and cell invasiveness. It has been demonstrated that MACC1 upregulates the expression of the *SLC9A1* gene, encoding for NHE1, thus enhancing its activity at plasma membrane. On the other hand, the electro-biochemical triggering begins with a resting potential depolarization due to the activity of $Na_V\alpha$ subunits promoting the activation and recruitment of the small GTPase Ras-related C3 botulinum toxin substrate 1 (Rac1) at the leading edge of migrating cells. Transforming growth factor beta 1 (TGF-β1) increases the expression levels of Na_V channels genes (*SCNxA*) while ring finger protein 1 (RING1B), RE1 silencing transcription factor (REST), histone deacetylase 2 (HDAC2) and salt inducible kinase 1 (SIK-1), as well as the n-3 polyunsaturated fatty acids n-3 (PUFA), repress their expression. SIK-1 also impairs the functioning of NHE1 exchanger. The "auxiliary subunit" $Na_V\beta4$ is expressed in normal epithelial cells but is importantly down-regulated in invasive cells and high-grade metastatic tumours. The absence of this protein, but specifically the lack

of the intracellular C-terminus domain, triggers the acquisition of an amoeboid-mesenchymal hybrid phenotype dependent of the small GTPase Ras homolog family member A (RhoA). $Na_V\beta1$ proteins have a dual role in cancer cells acting as cell adhesion molecules (CAMs) reducing cell migration and proliferation. However, it has also been demonstrated that $Na_V\beta1$ promotes tumour growth, metastasis and vascularization via the proto-oncogene tyrosine-protein kinase Fyn. The Rho-associated protein kinases (ROCK1/2) negatively regulate the expression of $Na_V\alpha$ subunits, therefore, silencing or inhibition of these repressors restore Na_V channels activity promoting an aggressive cell phenotype. Pharmacological intervention with FDA-approved drugs or new design small-molecule lead compounds against Na_V channels represent a promising strategy to decrease sodium channels-associated metastases.

Figure 3: Chemical structures of known $Na_V\alpha$ blockers with anticancer effects.

Table and legend

Table I: Expression and identified roles of pore-forming $Na_V\alpha$ and auxiliary $Na_V\beta$ in cancer

*Cancer tissue *vs* non-cancerous tissue

#Highly invasive cell line vs normal immortalized cell line

†In vitro

‡In vivo

Isoform	Expression levels	Cancer type	Role in cancer, proposed mechanism	References
$Na_V1.1$	Upregulated* (mRNA, protein)	Lymph nodes from CRC	Unknown, unknown	Lin et al., 2019
$Na_V1.2$	Upregulated* (Protein)	Liver	Unknown, unknown	The Human protein Atlas (www.proteinatlas.org)
$Na_V1.3$	Upregulated* (mRNA)	Ovarian	Unknown, unknown	Gao et al., 2010
$Na_V1.4$	Upregulated* (mRNA)	Cervix	Unknown, unknown	Diaz et al., 2007
$Na_V1.5$	Upregulated*# (mRNA, protein, I_{Na}^+)	Breast	↑Invasion†‡ ▪ Through an increased Src kinase activity promoting invadopodial formation and favoring an allosteric activation of NHE-1, extracellular acidification and enhanced activity of cysteine−cathepsins proteases ▪ Boosting the EMT transition via SIK1 ▪ Generating a sustained plasma membrane depolarization that leads to Rac1 activation and cytoskeleton reorganization ▪ Increasing MMP9 expression and reducing apoptosis	Roger et al., 2003, Fraser et al., 2005, Brackenbury et al., 2007, Gillet et al., 2009, Brisson et al., 2011, Yang et al., 2012, Brisson et al., 2013, Driffort et al., 2014, Nelson et al., 2015a,b, Gradek et al., 2019, Yang et al., 2020,
		Colorectal	↑Invasion† Through the regulation of the transcriptional pathway PKA/ERK/c-jun/ELK-1/ETS-1	House et al., 2010, Baptista-Hon et al., 2014, House et al., 2015, Guzel et al., 2019, Poisson et al., 2020

		Ovarian	↑Migrajon† ↑Invasion† ↑Proliferajon† ▪ By increasing the window current and depolarization of resting potential	Gao et al., 2010, Liu et al., 2018
		Leukemia	↑Invasion† ▪ Endosomal acidification and enhanced phagocytosis via calcium signaling	Fraser et al., 2004, Carrithers et al., 2007, Carrithers et al., 2012, Rahgozar et al., 2013, Jones et al., 2014, Huang et al., 2015
$Na_V1.6$	Upregulated*# (mRNA, protein, I_{Na}^+)	Cervix	↑Invasion† ▪ Boosted activity of MMP2 and NHE-1	Diaz et al., 2007, Hernandez-Plata et al., 2012, Lopez-Charcas et al., 2018
		Leukemia	↑Invasion† ▪ Invadopodial formation and enhanced activity	Carrithers et al., 2009
		Melanoma	↑Invasion† ▪ Invadopodial formation and enhanced activity	Carrithers et al., 2009
		Lymph nodes from CRC	Unknown, unknown	Lin et al., 2019
	Downregulated*# (mRNA, protein, I_{Na}^+)	Erwin sarcoma	↓Migrajon† ↑Apoptosis† Through a repression of *SCN8A* by RING1B and maintaining low NF-κβ levels	Hernandez-Munoz et al., 2016
		Colorectal	Unknown, unknown	Igci et al., 2015
$Na_V1.7$	Upregulated*# (mRNA, protein, I_{Na}^+)	Prostate	↑Invasion† ▪ Cell motility increased via galvanotaxis	Grimes et al., 1995, Diss et al., 2001, Diss et al., 2005, Yildirim et al., 2012, Bugan et al., 2019

		Lung (NSCLC)	↑Invasion[†] ▪ Dysregulation of sodium homeostasis ▪ Increase of $[Na^+]_i$ and depolarization of cell membrane	Roger et al., 2007 Campbell et al., 2013
		Lung (SCLC)	Unknown Participation in the formation of a neuroendocrine-like tumor cell phenotype	Pancrazio et al., 1989, Blandino et al., 1995, Onganer et al., 2005
		Stomach	↑Invasion[†‡] ↑Tumour growth[‡] ▪ Cancer progression through MACC1-mediated upregulation of NHE-1	Xia et al., 2016
		Endometrial	↑Invasion[†‡]↑Tumour growth[‡]↓Apoptosis[‡] ▪Unknown	Liu et al., 2018, Liu et al., 2019
	Downregulated* (mRNA, protein)	Colorectal	Unknown, unknown ▪ Proposed within a risk score with high prognostic value for overall survival	Pan et al., 2017
$Na_V1.8$	Upregulated[#] (mRNA)	Lung (NSCLC)	Unknown, unknown	Roger et al., 2007
$Na_V1.9$	Upregulated[#] (mRNA)	Lung (NSCLC)	Unknown, unknown	Roger et al., 2007
$Na_V\beta1$	Upregulated*[#] (mRNA, protein)	Prostate	Unknown, unknown	Diss et al., 2008
		Breast	↑Tumor growth[†‡] ↑Vascularizajon[‡] ↑Invasion[†] ↓Apoptosis ▪ β1 *trans-homophilic adhesion* triggering of cell process outgrowth via fyn kinase	Nelson et al., 2014, Bon et al., 2016
	Downregulated[#] (mRNA, protein)	Breast	↑Invasion[†] By decreasing cell adhesion and facilitating cell migration	Chioni et al., 2009
		Lung	↑Invasion[†] By decreasing cell adhesion	Campbell et al., 2013

		Cervix	↑Migration[†] ▪Acting as a cell adhesion molecule ↓Proliferation[†] ▪By increasing the population of cells in the G0/G1 phase in cell cycle	Sanchez-Sandoval and Gomora, 2019
$Na_V\beta2$	Upregulated[#] (mRNA, protein)	Prostate	↑Invasion[†] ▪ Promotion of bipolar cell morphology, enhanced cell adhesion and motility through association with neural substrates. ↓Tumour volume in xenograft models[‡]	Jansson et al., 2012, Jansson et al., 2014
$Na_V\beta3$	Upregulated[#] (mRNA, protein)	Bone	↑Apoptosis[†] ▪ Increase of a p53-dependent apoptotic pathway	Adachi et al., 2004
$Na_V\beta4$	Downregulated*[#] (mRNA, protein)	Breast	↑Invasion[†‡] ▪ Enhanced RhoA activity ▪ Mediated by the intracellular C-terminus motif ▪ Acquisition of a hybrid mesenchymal-amoeboid aggressive phenotype	Bon et al., 2016
		Lung	Unknown, unknown	Bon et al., 2016
		Cervix	↑Invasion[†]	Sanchez-Sandoval and Gomora, 2019
		Thyroid	Unknown ▪ Its expression is an indicator of favorable recurrence-free survival	Gong et al., 2018

Figure 1

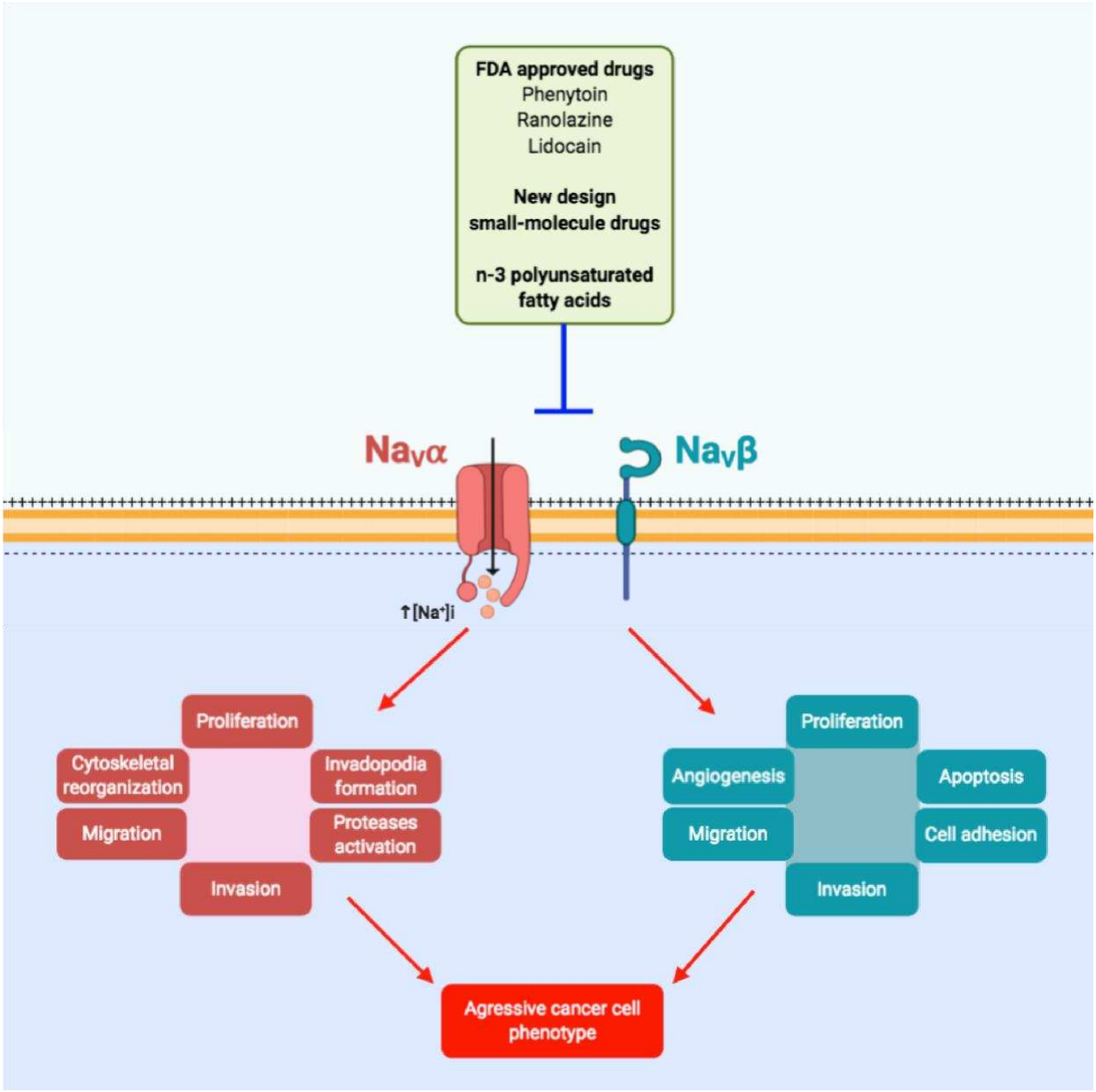
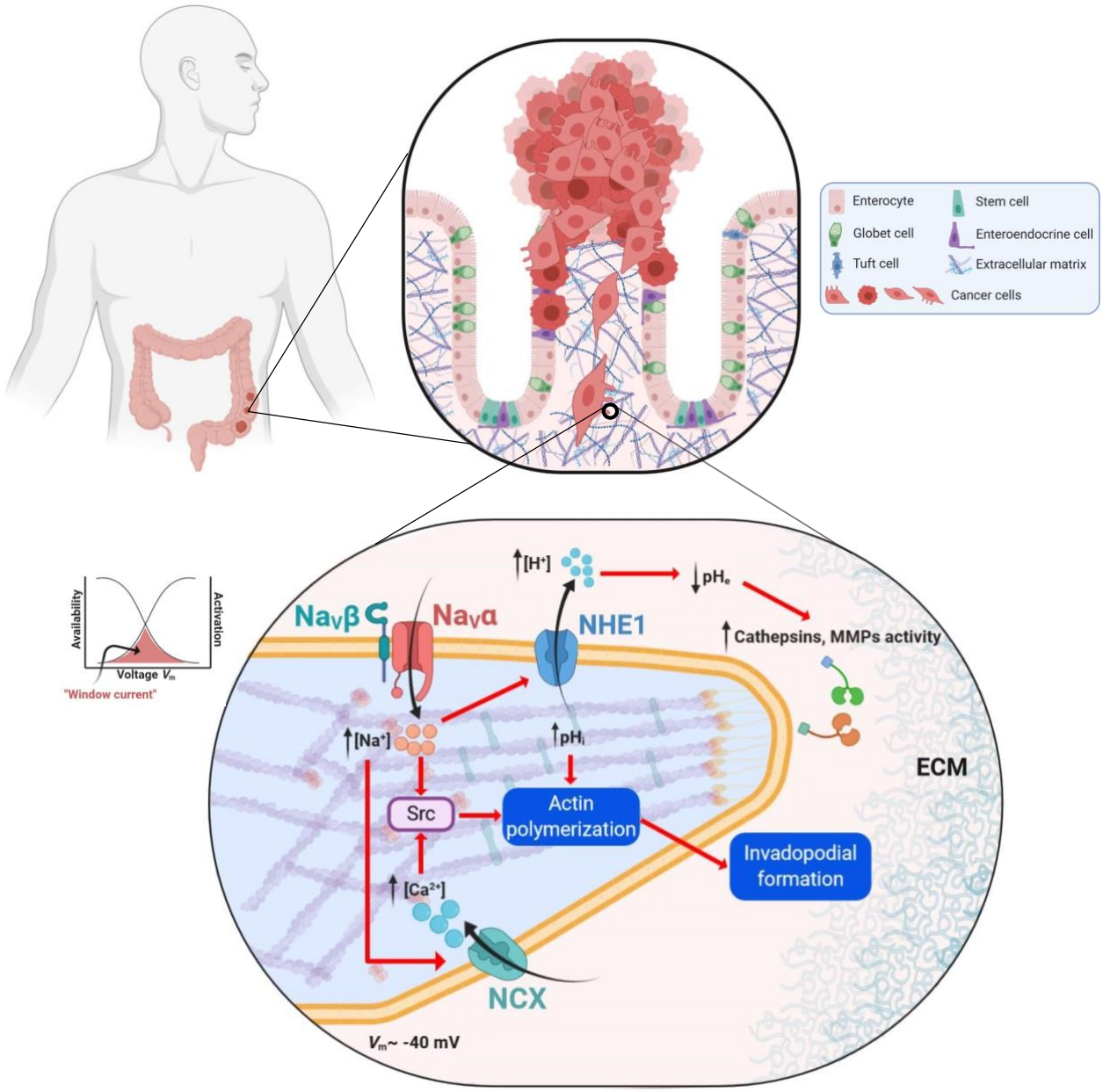

FDA approved drugs
Phenytoin
Ranolazine
Lidocain
New design small-molecule drugs
n-3 polyunsaturated fatty acids
Navα
Navβ
↑[Na+]i
Proliferation
Cytoskeletal reorganization
Invadopodia formation
Migration
Proteases activation
Invasion
Proliferation
Angiogenesis
Apoptosis
Migration
Cell adhesion
Invasion
Agressive cancer cell phenotype

Figure 2


Enterocyte
Stem cell
Globet cell
Enteroendocrine cell
Tuft cell
Extracellular matrix
Cancer cells
Availability
Activation
Voltage V_m
"Window current"
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$Na_V\alpha$
NHE1
[H+]
pH_e
Cathepsins, MMPs activity
[Na+]
pH_i
ECM
Src
Actin polymerization
Invadopodial formation
[Ca2+]
NCX
V_m~ -40 mV

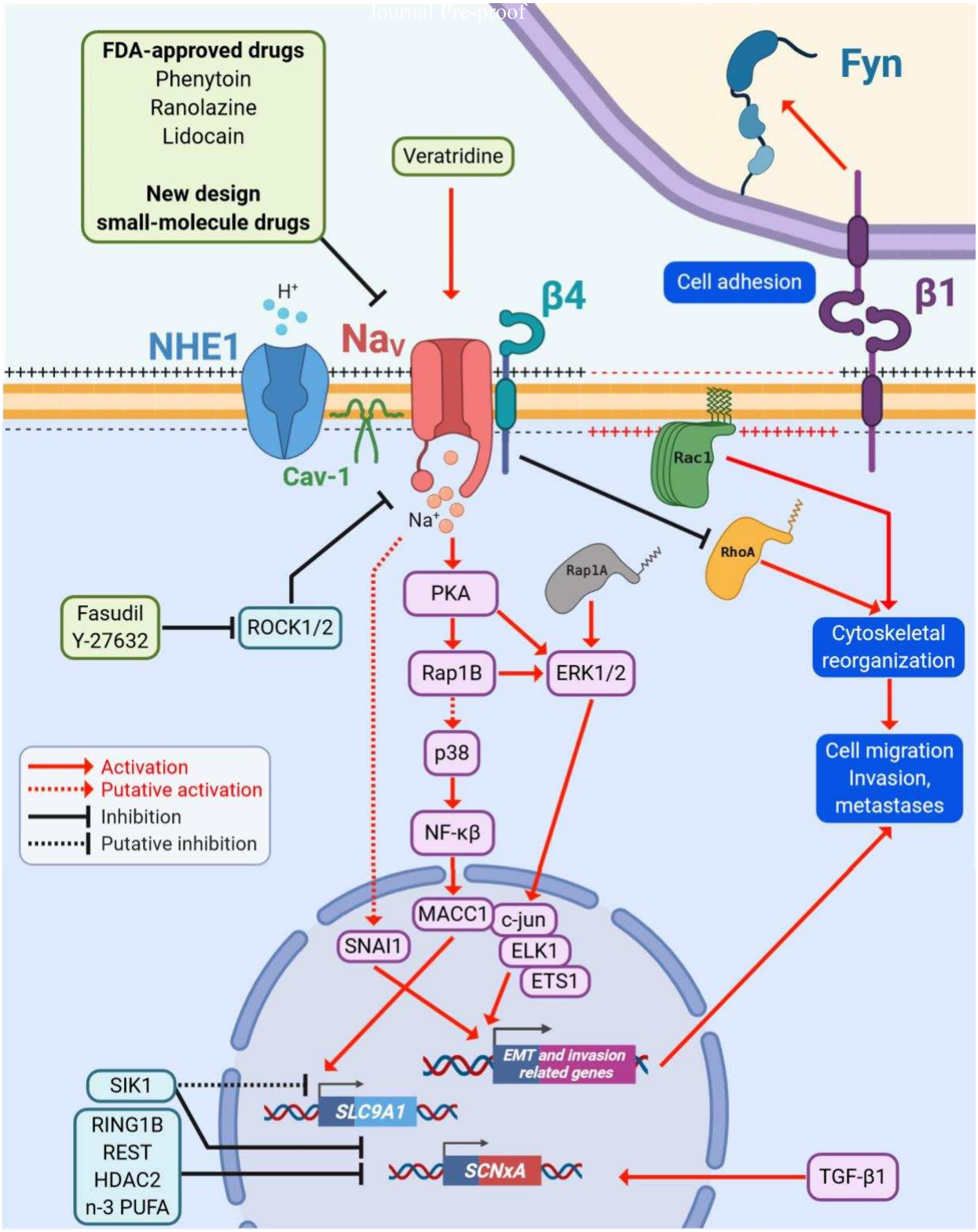
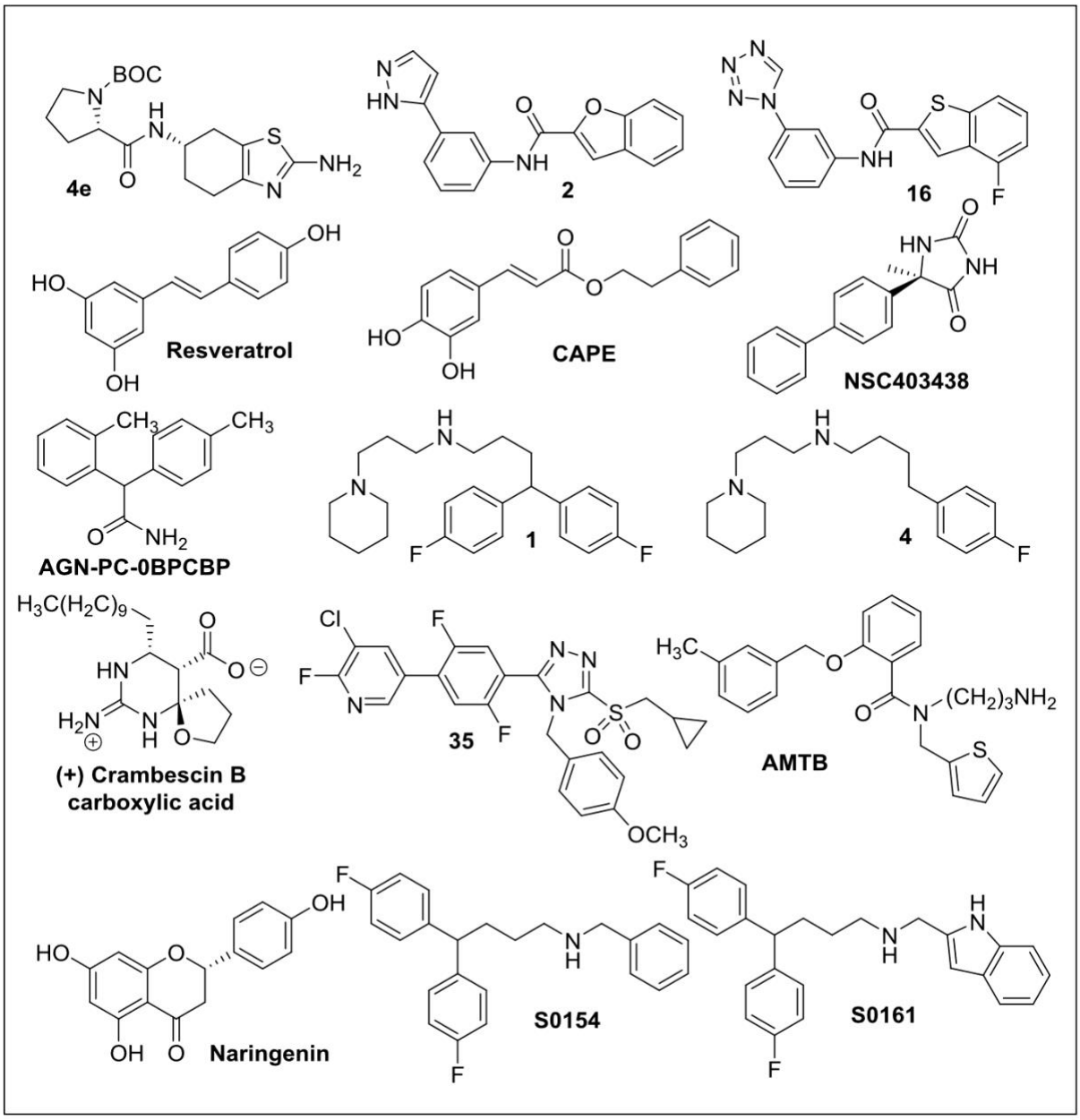


Figure 3


4e
2
16
Resveratrol
CAPE
NSC403438
AGN-PC-0BPCBP
1
4
(+) Crambescin B carboxylic acid
35
AMTB
Naringenin
S0154
S0161

Highlights

- Voltage-gated sodium (Na_V) channels are aberrantly expressed in cancers tissues
- Na_V activity promotes aggressive progression of cancer cells
- Na_V channels are pharmacological targets for anticancer treatments
- Na_V expression / activity could be modulated by nutritional interventions